RESEARCH PAPER

In Vitro Adme Evaluation of a Novel Secretory Phospholipase A_2 Inhibitor, Compound-9(O)

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ABSTRACT
Early evaluation of absorption, distribution, metabolism, and excretion (ADME) properties is critical in drug discovery to minimize late-stage failures associated with poor pharmacokinetics. In the present study, an integrated in vitro ADME assessment was conducted for Compound-9(O), a novel oxadiazole–triazole-based secretory phospholipase A_2 (sPLA$_2$) inhibitor previously identified as a potent candidate with superior activity compared to ursolic acid as reference inhibitor. Comprehensive ADME profiling, including kinetic solubility, Caco-2 permeability, plasma protein binding, metabolic stability, and cytochrome P450 (CYP) inhibition studies, was carried out to evaluate its developability. Compound 9(O) demonstrated favorable aqueous solubility and high intestinal permeability, suggesting that oral absorption is unlikely to be a limiting factor. However, extensive plasma protein binding indicates a reduced unbound fraction, which may affect pharmacological activity and systemic availability. Metabolic studies revealed moderate intrinsic clearance, highlighting the role of hepatic metabolism in compound elimination.

The CYP inhibition profile showed minimal inhibition of major isoforms such as CYP3A4 and CYP2D6, indicating a low risk of broad metabolic drug–drug interactions. However, significant inhibition of CYP2C19 was observed, representing a potential liability that requires further consideration.

Overall, the ADME profile of Compound-9(O) indicates a balance of favorable attributes, including solubility, permeability, and acceptable metabolic stability, alongside key challenges such as high plasma protein binding and CYP2C19 inhibition. These findings provide valuable insights for rational lead optimization through physicochemical modification, scaffold refinement, and formulation or prodrug strategies. The study highlights the importance of early ADME evaluation in bridging the gap between in vitro potency and in vivo pharmacokinetics, supporting the continued development of Compound-9(O) as a promising therapeutic candidate.

Keywords: In vitro ADME, Secretory phospholipase A_2 inhibitor, Plasma protein binding, Caco-2 permeability, Metabolic stability, CYP450 inhibition

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INTRODUCTION

In contemporary drug discovery, the identification of biologically potent compounds alone is insufficient for successful clinical translation. A significant proportion of drug candidates fail during development due to unfavorable absorption, distribution, metabolism, and excretion (ADME) properties rather than lack of efficacy. Consequently, early evaluation of in vitro ADME characteristics has become a critical component of lead optimization, enabling prediction of in vivo pharmacokinetics and early identification of developability risks [1].

In vitro ADME assays provide rapid, cost-effective, and mechanistically informative data that guide medicinal chemistry efforts and reduce later-stage attrition. Among these, kinetic solubility assessment is critical, as insufficient aqueous solubility can limit oral absorption and compromise bioavailability. Poor solubility is frequently associated with formulation challenges and inconsistent in vivo exposure, underscoring its importance in early-stage screening [1-3].

Srinivasa Rao Pedada and co-workers previously reported the design, synthesis, and biological evaluation of a novel series of secretory phospholipase A_2 (sPLA$_2$) inhibitors

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incorporating oxadiazole and triazole pharmacophores, which are commonly employed as bio-isosteres to enhance drug-like properties in medicinal chemistry [4, 5]. Among the synthesized derivatives, 1-((5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)methyl)-N-p-tolyl-1H-1,2,3-triazole-4-carboxamide, herein referred to as Compound-9(O), emerged as the most promising candidate. The compound exhibited potent inhibitory activity against $sPLA_2$, with an Inhibitory Constant 50% (IC_{50}) value of 8.51 ± 1.3 micromolar (μM), surpassing the activity of the standard reference inhibitor ursolic acid (IC_{50} = 12.59 ± 1.03 μM). Molecular docking studies further supported these findings by revealing favourable interactions of compound-9(O) within the PLA_2 active site, consistent with its observed enzymatic potency [5].

Based on its encouraging in vitro enzymatic inhibition and anticancer activity, compound-9(O) was selected for further investigation of its drug-like properties. In the case of compound-9(O), Although high intestinal permeability can be advantageous for absorption, it may not compensate for significant pre-systemic elimination or poor dissolution, ultimately resulting in low systemic exposure [2, 6]. Furthermore, inhibition of cytochrome P450 enzymes could lead to altered clearance mechanisms or drug–drug interaction liabilities, further influencing bioavailability and safety [7_ 9]

To better elucidate the rational optimization strategies, comprehensive in vitro absorption, distribution, metabolism, and excretion (ADME) investigations are warranted. Such studies, including kinetic solubility, plasma protein binding, Caco-2 permeability, metabolic stability, and CYP450 inhibition assays, are widely used to predict in vivo pharmacokinetics and to identify modifiable chemical liabilities at an early stage of development [10, 11]. Insights gained from these assays can guide physical or chemical modification of the molecule, formulation strategies, or prodrug approaches aimed at improving oral exposure.

Overall, in vitro ADME data provide a comprehensive understanding of the developability profile of Compound-9(O). Such an approach is critical for bridging the gap between in vitro potency and in vivo performance, and it facilitates informed decision-making for the further optimization and advancement of Compound-9(O) as a potential therapeutic candidate.

MATERIALS AND METHODS

Chemicals and reagents

Reagents and solvents were of analytical grade, acetonitrile (LC-MS grade) and methanol (LC-MS grade), Dimethyl Sulfoxide (HPLC grade) (DMSO) were purchased from Biosolve, France. Formic acid (suprapure grade) was purchased from Merck, Germany. type-1 water was purified in-house using a Milli-Q® Gradient A-10 system (Millipore Corporation, Bedford, MA, USA). Diclofenac sodium and Nicardipine hydrochloride, Verapamil, Ketoconazole, quinidine**,** sulfaphenazole, α naphthoflavone, benzylnirvanol, Midazolam, Dextromethorphan, Phenacetin, Mephenytoin**,** Imipramine and β-Nicotinamide Adenine Dinucleotide 2′- Phosphate (NADPH) was purchased from Sigma Aldrich Bangalore, India. Liver microsomes were purchased from Xenotech LLC (Kansas, USA).

Instrumentation

A Shimadzu N series LC-40D HPLC system (Kyoto, Japan) equipped with a binary pump (with a standard degasser), a well plate autosampler, and a column oven compartment, was used. Detection was performed using an SCIEX API 6500^+ (Concord, Ontario, Canada) mass spectrometer equipped with a Turbo Ion Spray (TIS) interface. A Kinetex, 2.6 μm, Biphenyl 100 A°, LC Column 100 X 4.6 mm, column was purchased from Phenomenex (Torrance, CA, USA).

METHODOLOGY

In vitro assays

Kinetic Solubility

The kinetic solubility of compound-9(O) was evaluated in sodium phosphate buffer (50 mM, pH 7.4) at 37 °C. Stock solutions of compound-9(O) were prepared in DMSO at concentrations ranging from 0.078 mM to 10 mM. For standard curve preparation, 5 μl of each stock solution was added to 995 μl of acetonitrile: buffer (1:1, v/v). For study samples, 10 μl of the respective stock solution was added to 990 μl of filtered sodium phosphate buffer. The samples were mixed well and incubated at room temperature (24 °C) for 120 min followed by mixing and centrifuged at 4000 rpm for 20 min. Supernatants were diluted 1:99 with acetonitrile containing internal standard and analyzed using the fit per purposes LC-MS/MS method. Diclofenac sodium and nicardipine hydrochloride were included as reference compounds [11,12].

Metabolic stability studies

In vitro studies were conducted using liver microsomes from male CD1 mice, male wistar rats, and human (mixed gender, Caucasian) liver microsomes to evaluate the metabolic stability of the novel compound. The reactions were performed in sodium phosphate buffer (pH 7.4) containing the necessary cofactors (NADPH) at 37 °C. Briefly, the novel compound concentration of 0.5 μM was incubated with liver microsomes at a final protein concentration of 0.5 mg/ml. Incubations were carried out for up to 30 min, with 50 μl aliquots collected at 0, 5, 10, 15, and 30 min and quenched with 150 μl acetonitrile to monitor parent compound degradation. For NADPH-free control incubation, samples were collected at 0 and 30 minutes. Verapamil was used as a positive control in mouse, rat, and human liver microsomes. Metabolic stability was evaluated by comparing the area ratio of the parent compound at each time point to determine the degradation rate. The compound's half-life ($t_{1/2}$) and intrinsic clearance (CL_{int}) were calculated by one phase exponential decay equation using GraphPad Prism® software [13].

Cytochrome P450 (CYP) Inhibition Studies

The inhibitory potential of compound-9(O) toward major human cytochrome P450 isozymes was evaluated using human liver microsomes. The study assessed inhibition of CYP3A4, CYP2D6, CYP2C9, CYP1A2 and CYP2C19 using probe substrates midazolam, dextromethorphan, diclofenac, phenacetin and mephenytoin and respectively. The compound-9(O) was incubated at increasing concentrations (up to 30 µM, based on visual solubility) with microsomes in the presence of NADPH at 37 °C. Reactions were terminated at predefined time points, and substrate metabolite formation was quantified using LC-MS/MS. Percent enzyme activity and percent inhibition were calculated relative to vehicle control.

Ketoconazole (CYP3A4), quinidine (CYP2D6), sulfaphenazole (CYP2C9), α-naphthoflavone (CYP1A2) and benzylnirvanol (CYP2C19) [14_16] were used as positive control inhibitors to confirm assay performance. IC_{50} values were derived from concentration–inhibition curves generated from two independent experimental sets. If IC_{50} values observed as IC50 < 1 µM – potent inhibitor, IC50 ≥ 1 µM to ≤ 10 µM – moderate inhibitor, IC50 > 10 µM – weak/ no inhibitor.

Caco-2 Cell Permeability Assay

The intestinal permeability of compound-9(O) was evaluated using a Caco-2 cell monolayer model. Caco-2 cells were seeded at a density of 80,000 cells/well and cultured until formation of a tight monolayer (after 21 days). Compound-9(O) was tested at a nominal concentration of 10 µM. Transport experiments were conducted in both apical-to-basolateral (A→B) and basolateral-to-apical (B→A) directions under physiological pH conditions (pH 7.4). Samples were collected over 120 min and analyzed by LC-MS/MS. Apparent permeability coefficients (P_app) were calculated using standard equations mentioned below. Efflux ratio was determined as the ratio of B→A to A→B permeability. Mass balance recovery was assessed at the end of the experiment to ensure compound stability [17].

Apparent permeability (Papp) was calculated using the formula:

$$P_{app} = \frac{dQ/dt}{A \times C_0}$$

dQ/dt - rate of test item or positive control in the receiver compartment

A - Surface area of the membrane (1.12 cm^2)

C0 - Initial concentration of test item or positive control

Efflux ratio = P_{app} BA/P_{app} AB

Plasma Protein Binding (PPB)

The plasma protein binding of compound-9 (O) was determined using the equilibrium dialysis method. The study was conducted using rat plasma collected from male rats and anticoagulated with K_2EDTA. The experiment was performed on a HT Dialysis system (HTD96b, Connecticut, USA) using cellulose dialysis membranes with a molecular weight cut-off of 12,000–14,000 Da [18-21].

Plasma samples were spiked with compound-9(O) at a final concentration of 3 µM and loaded into one side of the dialysis chamber, with sodium phosphate buffer (100 mM, pH 7.4) added to the opposite side. Each half-cell was loaded with a volume of 120 µl. The dialysis assembly was incubated at 37 °C with shaking at 60 rpm under a 5 % carbon dioxide atmosphere.

Samples were collected at 0 h and 5 h into pre-labelled microcentrifuge tubes. Compound concentrations were quantified using the LC-MS/MS method. Diclofenac was used as a reference compound. The percentage of unbound fraction (%fu), bound fraction (% bound), and recovery were calculated.

Statistical analysis

GraphPad Prism (version 9) was used for all the statistical calculations ADME assay experiments.

RESULTS AND DISCUSSION

In vitro assays

Kinetic Solubility

Compound 9(O) demonstrated good aqueous solubility in sodium phosphate buffer (pH 7.4), with a measured solubility of 100 µM, comparable to that of diclofenac sodium under the same conditions (Table 1). In contrast, nicardipine hydrochloride exhibited poor solubility (<0.78 µM), confirming the reliability of the assay.

Table 1.: Kinetic solubility results of compound-9(O)

Compound	Matrix	pH	LLOQ (µM)	Solubility (µM)
Compound-9 (O)	Sodium phosphate buffer	7.4	0.78	100
Diclofenac sodium	Sodium phosphate buffer	7.4	0.78	100
Nicardipine HCl	Sodium phosphate buffer	7.4	0.78	<0.78

Metabolic stability

The metabolic stability of Compound-9 (O) was evaluated in mouse, rat, and human liver microsomes. The compound exhibited species-dependent stability. In mouse microsomes, it showed low stability with a half-life of 11.4 min and high intrinsic clearance (122 µL/min/mg), indicating rapid metabolism. In contrast, the compound demonstrated improved stability in rat and human microsomes, with half-life values greater than 30 min. The intrinsic clearance was moderate in rat (35 µL/min/mg) and very low in human microsomes (2.5 µL/min/mg), suggesting favorable metabolic stability, particularly in humans.

The positive control, verapamil, showed high clearance across all species, confirming the validity of the assay. The

intrinsic clearance values of the compound and positive control are mentioned in Table 2 and Table 3., respectively

Table 2.: Average intrinsic clearance and half-life values of compound 9 (O) in mouse, rat, and human liver microsomes

Positive Control Substrate	Liver Microsomes	0.5 mg/ml protein concentration	
		Half-life (min)	CLint (µl/min/mg protein)
Compound-9(O)	Mouse	11.4	122
	Rat	>30	35
	Human	>30	2.5

Table 3.: Intrinsic clearance and half-life values of positive control substrate verapamil in mouse, rat, and human liver microsomes

Positive Control Substrate	Liver Microsomes	0.5 mg/ml protein concentration	
		Half-life (min)	CLint (µl/min/mg protein)
Verapamil	Mouse	4.6	299
	Rat	18.0	77
	Human	20.1	69

CYP450 Inhibition

Compound-9(O) demonstrated minimal inhibitory activity toward major CYP450 isoforms involved in drug metabolism. The summary result of CYP450 Inhibition for compound-9(O) is mentioned in Table 4

CYP3A4 (Midazolam)

Compound-9(O) showed weak inhibition of CYP3A4, with IC_{50} values greater than 30 µM in two independent runs. At the highest concentration tested (30 µM), enzyme inhibition ranged from 34–46%, indicating low interaction potential. In contrast, the positive control ketoconazole showed potent inhibition (IC_{50} ≈ 0.012 µM), confirming assay sensitivity.

CYP2D6 (Dextromethorphan)

For CYP2D6, compound-9 (O) exhibited less than 35% inhibition even at 30 µM, resulting in IC_{50} values >30 µM. Quinidine, used as the reference inhibitor, showed expected strong inhibition (IC_{50} ≈ 0.03 µM).

CYP2C9 (Diclofenac)

Moderate to high inhibition was observed against CYP2C9, with IC_{50} values of 18.24 µM and 24.23 µM across two independent experiments. Although weaker than sulfaphenazole (IC_{50} ≈ 0.2 µM), the observed inhibition suggests a moderate to high interaction potential at supratherapeutic concentrations.

CYP1A2 (Phenacetin)

Compound-9(O) exhibited weak inhibition of CYP1A2. The estimated IC_{50} values were 69 µM and 40 µM (no or weak inhibition) across two independent experimental sets, indicating low affinity toward this isozyme. At therapeutically relevant concentrations, CYP1A2 inhibition was minimal.

In contrast, the positive control α-naphthoflavone showed potent inhibition with an IC_{50} of 0.005 µM, confirming the robustness and sensitivity of the assay. The results suggest that compound-9(O) is unlikely to cause clinically significant CYP1A2-mediated drug–drug interactions.

CYP2C19 (Mephenytoin)

Compound-9(O) demonstrated strong inhibition of CYP2C19, with consistent IC_{50} values of 0.28 µM in both independent experimental sets. This inhibitory potency was comparable to the reference inhibitor benzylnirvanol, which exhibited IC_{50} values ranging from 0.34–0.37 µM.

The observed CYP2C19 inhibition (potent inhibition, <1 µM) indicates a high interaction potential for this isozyme and suggests that co-administration of compound-9(O) with drugs primarily metabolized by CYP2C19 may require careful consideration during further development.

Table 4.: The summary result of CYP450 inhibition for compound-9(O)

CYP Isozyme	Probe Substrate	IC_{50} (µM) – compound-9(O)	Reference Inhibitor (IC_{50}, µM)	Interaction Risk
CYP1A2	Phenacetin	40–69	α-Naphthoflavone (0.005)	Low
CYP2C19	Mephenytoin	0.28	Benzylnirvanol (0.34–0.37)	High
CYP2C9	Diclofenac	18–24	Sulfaphenazole (~0.2)	Low–moderate
CYP2D6	Dextromethorphan	>30	Quinidine (~0.03)	Low
CYP3A4	Midazolam	>30	Ketoconazole (~0.01)	Low

Caco-2 Permeability

Compound-9(O) demonstrated high intestinal permeability in the Caco-2 cell monolayer model. The apparent permeability coefficient (P_app) in the apical-to-basolateral direction was 20.5 ×10^{-6} cm/s, which is characteristic of highly permeable compounds. The B→A permeability was lower (14.1 ×10^{-6} cm/s), resulting in an efflux ratio of 0.69, indicating no involvement of active efflux transporters such as P-gp. Mass balance recovery was acceptable, with 99% recovery in the A→B direction and 80% recovery in the B→A direction, confirming compound stability under assay conditions.

The apparent permeability and efflux ratio values for the test compound and positive controls are presented in the following Table5 and Table 6, respectively.

Table 5.: The apparent permeability and efflux ratio values for compound-9(O)

Name of the Compound	compound-9(O)	
Parameter	**A → B**	**B → A**
P_app (nm/s)	205 ± 37	141 ± 8
P_app (×10⁻⁶ cm/s)	20.5	14.1
Efflux ratio	0.69	
% Recovery	99	80

Table 6.: The apparent permeability and efflux ratio values for positive controls

Compound	Atenolol		Metoprolol	
Transport	**A to B**	**B to A**	**A to B**	**B to A**
P_{app} (nm/s)	3.23 ± 0.31	7.79± 2.51	250.41 ± 125.59	138.39 ± 10.39
Efflux Ratio	2.14		0.60	
% Recovery	**99**	**102**	**127**	**93**

The permeability of compound-9(O) across the Caco-2 cell monolayer was found to be high (Papp >100 nm/sec). The reference compounds, atenolol (low permeability marker) and metoprolol (high permeability marker), exhibited permeability values of <10 nm/sec and >100 nm/sec, respectively, which were consistent with historical data. The efflux ratio of compound-9(O) was less than 2, indicating that it is primarily transported via passive diffusion and is not a substrate of the P-glycoprotein (P-gp) efflux transporter. Additionally, the transepithelial electrical resistance (TEER) values of the monolayer remained above 300 Ω·cm² both before and after the experiment, confirming the integrity of the cell monolayer. The permeability results for the control compounds were also in agreement with established literature values. Furthermore, the mean recovery values for compound-9(O) and the control compounds ranged between 80% to 127%, indicating that acceptable mass balance was achieved during the assay. The high permeability observed suggests that intestinal absorption is not a limiting factor for compound-9(O).

Plasma Protein Binding

Compound-9(O) exhibited high plasma protein binding in rat plasma, with an unbound fraction of 0.49 %, indicating that approximately 99.51 % of the compound was bound to rat plasma proteins. The recovery of the compound was 87 %, confirming acceptable mass balance across the dialysis system. The results summary of PPB for compound-9(O) and reference compound is mentioned in below Table 7.

Diclofenac, used as a reference compound, showed a comparable unbound fraction (0.55 %) and recovery (103 %), validating the performance of the assay. The high plasma protein binding of compound-9(O) is consistent with its lipophilic scaffold and may contribute to its pharmacokinetic behavior, including limited free circulating concentration.

Table 7.: The results summary of PPB for compound-9(O)

Compound	Species	Concentration (µM)	% Unbound	% Bound	% Recovery
Compound-9(O)	Rat	3.0	0.49	99.51	87
Diclofenac	Rat	3.0	0.55	99.45	103

CONCLUSION

In the present study, the integrated in vitro ADME evaluation of Compound 9(O) provided comprehensive mechanistic insights into its pharmacokinetic profile. The compound exhibited favorable aqueous solubility and high intestinal permeability, indicating that oral absorption is unlikely to be a limiting factor for systemic exposure.

However, extensive plasma protein binding is expected to significantly reduce the unbound fraction available for pharmacological activity and systemic clearance. In addition, the observed intrinsic clearance in liver microsomes suggests that hepatic metabolism plays a notable role in the elimination of the compound.

The CYP inhibition profile revealed minimal inhibitory effects on major isoforms such as CYP3A4 and CYP2D6, indicating a generally favorable metabolic interaction profile. Nevertheless, consistent and potent inhibition of CYP2C19 highlights a potential drug–drug interaction liability that must be carefully considered in further development.

Collectively, these findings identify key determinants governing the disposition of Compound-9(O). While the compound demonstrates favorable solubility, permeability, and metabolic stability, high plasma protein binding and selective CYP2C19 inhibition emerge as critical liabilities that may impact bioavailability and safety.

These insights provide a strong foundation for rational lead optimization, including physicochemical modification, scaffold refinement, and formulation or prodrug strategies aimed at improving systemic exposure without compromising biological activity.

Overall, this study underscores the importance of early in vitro ADME evaluation in bridging the gap between in vitro potency and in vivo pharmacokinetic performance. The results support the continued development of the oxadiazole–triazole scaffold represented by Compound 9(O), while clearly outlining the optimization strategies necessary for advancing this promising PLA_2 inhibitor toward successful preclinical development.

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Authors contributions
Nageswararao P. - Methodology, Conceptualization, Experimentation, Validation, Writing Original draft, visualization, Resources, Project administration; Bhavesh B Patel – Data curation, Software, Resources, Project administration, Conceptualization Writing, review, and editing; Surendra B- Experimentation, Review, writing, Editing; Paras Tak-Supervision, Resources, Project administration, Conceptualization.

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