Synthesis, molecular modelling, *in vitro* and *in vivo* evaluation of conophylline inspired novel benzyloxy substituted indole glyoxylamides as potent pancreatic lipase inhibitors

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**ABSTRACT**

Pancreatic lipase is a digestive enzyme involved in the hydrolysis of dietary fats. Orlistat, a potent pancreatic lipase inhibitor, is widely prescribed for long-term obesity treatment. Nevertheless, orlistat is reported for severe adverse effects including hepatotoxicity and pancreatitis. In the present study, a novel series of 11 benzyloxy substituted indole glyoxylamides were designed, synthesized and evaluated for *in vitro* pancreatic lipase inhibitory activity. Three analogues, 10b, 11b and 11c, exhibited potent activity (IC50 ≤ 2.5 μM), with 11b exhibiting a potent IC50 of 1.68 μM comparable to orlistat (IC50 = 0.99 μM). Further, 11b exhibited reversible competitive inhibition with an inhibitory constant value of 0.98 μM. Molecular docking of these analogues was in agreement with *in vitro* results, wherein the MolDock scores exhibited significant correlation with their inhibitory activity (Pearson’s r = 0.7122). A 50 ns molecular dynamics simulation of 11b-pancreatic lipase complex confirmed the role of extended alkyl interactions along with π−π stacking and π-cation interactions, in stabilizing the ligand (Maximum RMSD ≈ 3 Å) in the active site. Gastro-intestinal absorption and toxicity prediction of the three potent analogues highlighted the suitability of 11b for *in vivo* experiments. 11b at a dose of 20 mg/kg exhibited anti-obesity efficacy comparable to orlistat (10 mg/kg), wherein the serum triglycerides were found to be 94.95 and 83.85 mg/dL, respectively. Further, faecal triglyceride quantification indicated 11b to act through pancreatic lipase inhibition similar to orlistat. The present study identified a novel pancreatic lipase inhibitory benzyloxy substituted bis(indolyl) glyoxylamide 11b, with promising anti-obesity activity.

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1. Introduction

Obesity is a chronic, relapsing metabolic disease characterized by excessive fat deposits in the body (Bray et al., 2017; Grundy, 1998; [http://www.who.int/topics/obesity/en/]). As per the recent WHO statistics, over 650 million adults were found to be obese, accounting to 13% of global population ([http://www.who.int/mediacentre/factsheets/fs311/en/](http://www.who.int/mediacentre/factsheets/fs311/en/)). While obesity is preventable, a chronic condition might lead to severe comorbid risks including diabetes mellitus (type II) and heart disease (Khaodhiar et al., 1999). Orlistat ([Figure 1](#fig1)), a potent pancreatic lipase (PL) inhibitor, is one among few drugs, widely prescribed for long term treatment of obesity. The β-lactone warhead of orlistat exhibits reversible covalent inhibition on the active site Ser152, while the long hydrophobic chains interact with the lid domain amino acids viz., Gly76 - Phe80 and Leu213 - Met217 of PL (Hadvary et al., 1991). While orlistat was reported to exhibit tolerable side effects, recent reports from FDA has cited severe adverse effects with long term administration of orlistat including hepatotoxicity and acute pancreatitis ([https://www.nlm.nih.gov/medlineplus/druginfo/meds/a601244.html#side-effects;](https://www.nlm.nih.gov/medlineplus/druginfo/meds/a601244.html#side-effects;)[https://wayback.archive-it.org/7993/20170112172652/http://](https://wayback.archive-it.org/7993/20170112172652/http://))
www.fda.gov/Safety/MedWatch/SafetyInformation/ucm215504.htm). These events highlighted the necessity for the quest of safer and effective anti-obesity drugs.

In recent years, various amide functionality-based pharmacophores, viz., α-ketoamides, tripeptides, 1,3-diketoamides and rhodanines, have been reported to exhibit potential PL inhibition (Chauhan et al., 2019; Chiu et al., 2000; Kokotos et al., 2000; Kotsovolou et al., 2001; Sridhar, Bhurta, et al., 2017; Stefanucci, Dimmito, et al., 2019; Stefanucci, Luisi, et al., 2019). The carbonyl groups of these amides mimic those of the natural esters in triglycerides, thus acting as electrophiles for Ser152 of the active site of PL. In particular, scaffolds with 1,3-diketoamide and α-ketoamide functionality possessed greater PL inhibitory potential, owing to the greater reactivity of their carbonyl groups (Han et al., 2000; Steuer et al., 2011).

Previously, we have identified conophylline (2, Figure 1) as a potential PL inhibitory natural product lead from Tabernaemontana divaricata R.Br. ex Roem. & Schult. (Sridhar et al., 2017). Conophylline, a bis-indole alkaloid, exhibited an IC$_{50}$ of 3.31 µM, however, displayed a lower potential in comparison to orlistat (IC$_{50}$ = 0.99 µM), due to its structural rigidity, and the lack of a highly reactive carbonyl group in contrast to orlistat (Figure 1). Consequent studies that involved scaffold hopping resulted in various conophylline inspired α-ketoamide analogues with potential PL inhibitory activity (Sridhar, Ginson, et al., 2017; Sridhar et al., 2019, 2020). In addition, these studies also highlighted the pivotal role of π–π and alkyl interactions with the lid domain, and π-cation interactions with Arg256 for potential PL inhibition (Figure 2). The most potent analogue of these series, an N-geranyl substituted bis(indolyl) oxoacetamide (5), exhibited greater PL inhibitory potential (IC$_{50}$ = 2.95 µM) in comparison to conophylline (2).

The present study aimed at further lead optimization of analogue 5 to enhance its PL inhibitory potential. Preliminary in silico studies as well as the results from our previous studies indicated benzyloxy substitution on the benzene ring of the indole, and the presence of a carbon linker between the amide and the aryl extension (Ar) to be favourable for enhancing π–π interactions with the lid domain and π-cation interaction with Arg256, respectively (Figure 3). Accordingly, a novel series of 11 benzyloxy indole glyoxylamides were designed, synthesized and evaluated for in silico and in vitro PL inhibitory activity. Further, the most potent analogues were predicted for their toxicity, while the anti-obesity efficacy of the most potent analogue, 11b, was studied using a pre-clinical anti-obesity mice model.

2. Materials and methods

2.1. Synthesis and characterization

The synthesis of all the final analogues were carried out as per the procedure detailed in Scheme 1. Progress of the reactions was followed by thin layer chromatography (TLC) analysis (Silica gel G60 F$_{254}$, Merck). Melting points were determined with electro thermal capillary melting point apparatus (E-Z melting) and are uncorrected. IR spectra were recorded on Shimadzu IRPrestige 21 FTIR machine using KBr pellets. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker Avance II 400 spectrometer (400 MHz and 100 MHz, respectively) using DMSO-$d_6$ and CDCl$_3$ as solvent. HRMS analysis was performed on Bruker Compass Data Analysis 4.1 mass instrument (See Supplementary Data).

2.1.1. General procedure for the synthesis of N-geranyl substituted benzyloxyindole (7)

Briefly, to 10 mL DMF taken in a 100 mL round bottomed flask, 25.6 mmol of KOH was added and stirred vigorously for 15 min at room temperature. To this, 8.5 mmol of various benzyloxy substituted indoles (6) was added and the stirring
was continued. After 30 min, a solution of geranyl bromide (8.5 mmol in 5 mL DMF) was added dropwise for over 5 min to the reaction mixture, and the stirring was continued overnight. The reaction mixture was then transferred to ice-cold water. The N-geranyl derivatives of various benzyloxy substituted indoles, obtained as oily liquids, were extracted using chloroform and purified through column chromatography.

2.1.2. General procedure for the synthesis of indole glyoxylamides

Various N-geranylated benzyloxyindole analogues (7, 2.5 mmol) were stirred at 0°C for 30 min in 5 mL THF. To this, oxalyl chloride (2.5 mmol) was added dropwise for a period of over 5 min, and the reaction was continued at 0°C. The obtained crude precipitate of glyoxyl chloride derivative (8) was dried in vacuo to remove the excess oxalyl chloride, and then transferred immediately to a solution of THF containing appropriate amount of various amine derivatives, followed by addition of triethylamine (7.5 mmol). The reaction was continued at room temperature for 2 h, and THF was removed in vacuo to yield crude benzyloxy substituted indole glyoxylamides. The obtained crude precipitate was treated with saturated solution of aqueous sodium bicarbonate, dried and then re-crystallized from ethanol to obtain the final compounds in 55–79% yields.

2.2. Pancreatic lipase inhibition and kinetics

Orlistat (1), Porcine PL (Type II) and 4-nitrophenyl butyrate were procured from Sigma-Aldrich (MO, USA). Tris buffer and sodium chloride (molecular biology grade) were procured from Sisco Research Laboratories (MH, India). All other
chemicals and solvents (analytical grade) were used without further purification.

The procedure for PL inhibition assay and kinetics was performed as per the protocol standardized in our laboratory (Sridhar et al., 2020). Briefly, a suspension of porcine PL (5 mg/mL) was subjected to vigorous shaking, followed by centrifugation (4000 rpm, 10 min), and the supernatant was used afresh as the enzyme solution. Stock solutions of the synthesized compounds and orlistat (1) were prepared in DMSO at linear concentrations ranging from 0.39–250 μg/mL. The final reaction mixture comprised of 875 μL of buffer, 100 μL of enzyme and 20 μL of the compounds of various stock concentrations, pre-incubated for 5 min at 37°C, followed by addition of 5 μL of the substrate (4-nitrophenyl butyrate, 10 mM in acetonitrile). The absorbance of the final mixture was taken on a BioTek EPOCH microplate spectrophotometer (VT, USA) after 5 min at absorbance maxima of 4-nitrophenol (405 nm). The assay was performed in triplicate and the percentage inhibition was calculated using the formula

\[ \%\text{inhibition} = \left(1 - \frac{A_T}{A_E}\right) \times 100 \]

where \( A_E \) is the absorbance of enzyme control (without inhibitor), and \( A_T \) is the difference between the absorbance of test sample, with and without substrate. The IC_{50} of the compounds was calculated by plotting linear regression curve.

For the inhibition kinetics, the assay protocol was repeated with varying concentrations (0, 5 and 10 μM) of 11b and substrate (25, 50, 100 and 200 μM), and a double reciprocal Lineweaver-Burk plot was plotted to understand the nature of inhibition (Lineweaver & Burk, 1934). The inhibition constant, \( K_i \), was calculated using Cheng-Prusoff equation (Burlingham & Widlanski, 2003).

### 2.3. Molecular docking

Molecular docking of the novel benzylxyo substituted indole glyoxylamides was performed using Molegro Virtual Docker 6.0 (Thomsen & Christensen, 2006). Prior to docking, all the ligands were energy minimized using Molecular Mechanics 2 (MM2) force field in Chem3D module of ChemBioOffice v12 (PerkinElmer, USA). For the crystal structure of Human PL, only two PDBs were reported to date, 1N8S and 1LPB (Egloff et al., 1995; van Tilbeurgh et al., 1992). Of these, 1N8S corresponds to the closed lid conformation of the Human PL, while 1LPB corresponds to the open lid conformation. Hence, the crystal structure of 1LPB was retrieved from the RCSB PDB Data bank, and the energy minimized ligands were docked in to the active site using previously validated grid parameters (Sridhar et al., 2019).

### 2.4. Molecular dynamics simulation

Molecular dynamics (MD) simulation of analogue 11b in complex with PL was performed for 50 ns as per the protocol standardized in our laboratory (Sridhar, Mutya, et al., 2017). Gromacs 5.0.4, compiled on a CentOS 7 operating system equipped with Intel(R) Xeon(R) CPU W3565 and NVIDIA Quadro 4000 Quad-Core Processor was used for the purpose of MD simulation (Abraham et al., 2015). CHARMM27 force field (MacKerell et al., 2000) was applied during the MD run, and the topology of the ligand 11b was generated using online tool provided by Swiss Institute of Bioinformatics (Zoete et al., 2011). Prior to the initiation of the MD, the complex was minimized using Steepest Descent algorithm for 1000 steps, followed by stabilization of the system to 310 K and 1 atm pressure for 50 ps, using the canonical NVT and NPT ensembles. Parameters like Particle Mesh Ewald method for long-range electrostatics (Darden et al., 1993), and 14 Å cut-off for van der Waals and columbic interactions were set during the MD simulation. LINCS algorithm was applied for the calculation of bond length (Hess et al., 1997). Discovery Studio 4.5 visualizer (Accelrys, USA) was used to depict the graphical representations of the complex.

### 2.5. Gastro-intestinal (GI) absorption and toxicity prediction

The GI absorption and toxicity profiles of the most potent analogues, 10b, 11b and 11c, were predicted alongside orlistat (1) and analogue 5 using various online tools and freeware. Parameters such as consensus LogP_O/W and GI absorption were predicted using SwissADME (Daina et al., 2017), while toxicity parameters including oral toxicity (predicted as LD_{50}) and hepatotoxicity were predicted using ProTox-II (Banerjee et al., 2018). Carcinogenicity parameters, that included both genotoxicity and non-genotoxicity were predicted using ToxTree v3.1.0 (Benigni et al., 2008).

### 2.6. In vivo experiments

#### 2.6.1. Animals and diets

A total of 54 male Swiss albino mice (15–20 g) were purchased from the Central Animal Facility of Birla Institute of Technology and Science, Pilani (BITS Pilani), Pilani Campus (India) (Registration number: 417/PO/ReBi/2001/CPCSEA). The mice were housed in polycyacrylic cages and maintained under standard husbandry conditions (room temperature 22 ± 1°C and relative humidity of 60%) with 12 h light/dark cycle. The animals were fed with either NPD or HFD (HFD) and filtered water ad libitum. The formula for high-fat diet used for the study was prepared as per previous literature (Shi et al., 2014) and contained 20% protein, 45% lipids and 35% carbohydrates by weight (Table S1, Supplementary Data).

#### 2.6.2. Experimental protocol

Animals were treated according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals and all the experimental procedures on animals were in compliance with the Institutional Animal Ethics Committee of BITS Pilani (protocol No: IAEC/RES/20/06/Rev-2/24/20). Briefly, three studies were conducted, namely (a) Oral Triglyceride Tolerance Test, (b) Long term (4-
2.6.2.2. Long term (4-week) treatment study. The effect of 11b on fat accumulation was examined by administering the drug to HFD fed mice over a period of 4 weeks (Avci et al., 2010). Prior to the treatment, all the animals under HFD groups were adapted to the HFD for a period of 2 weeks. After the adaptation period, the animals were divided into five groups (n = 6) as summarized in Table 1. For the treatment groups, orlistat or 11b were dissolved in 3% v/v Tween-80 solution and administered to the animals with an oral gavage, and the treatment was continued for 4 weeks.

The body weights of the animals were recorded weekly and were subjected to an overnight fast at the end of every week, followed by the collection of blood samples using the retro-orbital puncture. The blood samples were then centrifuged (1500 g) to obtain the serum. Various serum biochemical parameters including glucose, triglycerides, total cholesterol and HDL-cholesterol were estimated using commercially available diagnostic kits (Spinreact S.A.U., Spain), while LDL-cholesterol and HDL-cholesterol were estimated using column chromatography. The N-geranylated benzyloxyindole analogues (7) were then allowed to react with oxalyl chloride under ice-cold conditions to afford the respective glyoxyl chloride derivative 8 in good yields. The reaction time for the synthesis of the glyoxyxl chloride (8) varied with the benzyloxy substitutions. For instance, the reaction time for 4- and 6-benzyloxy analogues was around 3 h, while this time has been extended to 1 h and 3 h, respectively, for 5-benzyloxy and 7-benzyloxy analogues (Sridhar et al., 2020; Sridhar et al., 2020). The reaction time was varied with the benzyloxy substitutions. For instance, the reaction time for 5- and 6-benzyloxy analogues was around 30 min, while this time has been extended to 1 h and 3 h, respectively, for 5-benzyloxy and 7-benzyloxy analogues (Acar et al., 2010; Acar et al., 2010). Finally, the glyoxyxl chloride analogues (8) were made to react either with 3,4,5-trimethoxyaniline/3,4,5-trimethoxybenzylamine/tryptamine to yield the final analogues, 9a–d, 10a–c and 11a–d, respectively. All these analogues were synthesized for the first time as confirmed via SciFinder.

2.6.2.3. Quantification of faecal triglycerides. The procedure for quantification of faecal triglycerides was performed as per the literature report with minor modifications (T. Y. Chen et al., 2018). Briefly, 1 g of faeces was taken in a separatory funnel and subjected to vigorous shaking in 0.15 M NaCl. To this suspension, chloroform: methanol (4:1 v/v) was added and the shaking was continued. The mixture was allowed to separate and the lower organic phase was then collected, filtered and dried in vacuo. The obtained triglycerides were then dissolved in 1 mL ethanol, and the quantity of triglycerides was estimated using commercial kit (Spinreact S.A.U., Spain).

2.6.3. Statistical analysis

All the data were represented as mean ± SEM, and the differences were analysed using one-way analysis of variance (ANOVA) followed by post-hoc analysis of Tukey’s multiple comparison test to determine significant differences between the groups. Statistical calculation was performed using GraphPad Prism (v.5.0). A level of p < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Synthesis and characterization

The syntheses of all the final compounds (Table 2) were carried out as per the procedure represented in Scheme 1 (Guggilapu et al., 2017; Sridhar et al., 2020). Various benzyloxy substituted indole analogues (6) were subjected to N-geranylation and the resulting products (7), obtained as oily liquids, were extracted using chloroform and purified through column chromatography. The N-geranylated benzyloxyindole analogues (7) were then allowed to react with oxalyl chloride under ice-cold conditions to afford the respective glyoxyxl chloride derivative 8 in good yields. The reaction time for the synthesis of the glyoxyxl chloride (8) varied with the benzyloxy substitutions. For instance, the reaction time for 4- and 6-benzyloxy analogues was around 3 h, while this time has been extended to 1 h and 3 h, respectively, for 5-benzyloxy and 7-benzyloxy analogues (Sridhar et al., 2020). The reaction time was varied with the benzyloxy substitutions. For instance, the reaction time for 5- and 6-benzyloxy analogues was around 30 min, while this time has been extended to 1 h and 3 h, respectively, for 5-benzyloxy and 7-benzyloxy analogues (Acar et al., 2010; Acar et al., 2010). Finally, the glyoxyxl chloride analogues (8) were made to react either with 3,4,5-trimethoxyaniline/3,4,5-trimethoxybenzylamine/tryptamine to yield the final analogues, 9a–d, 10a–c and 11a–d, respectively. All these analogues were synthesized for the first time as confirmed via SciFinder.

The structures of the synthesized analogues were well characterized using NMR (1H and 13C), HRMS and IR (See Supplementary Data). In the 1H NMR, the –NH proton of the amide resonated at 8-9 ppm with. Further, the amide –NH appeared as a triplet due to the adjacent –CH3 protons in the trimethoxybenzyl and 2-(1H-indol-3-yl)ethyl substituted analogues, 10a–c and 11a–d, with few exceptions. The –CH2 protons of the benzyloxy extension resonated at 5-6 ppm, while the –OCH3 protons on the aryl substitution of the amide for analogues 9a–d and 10a–c resonated as singlets at 3-4 ppm. Further, the terminal –CH3 protons of the N-geranyl substitution resonated at 1.5-2 ppm. In 13C NMR spectra, carbons of carbonyl and amide functionalities resonated at ~180 (CO) and ~160 ppm (NHCO). In addition, the three methyl carbons as well as the carbons of the –CH2–CH2– linkers of the N-geranyl substitution resonated in between 16

Table 1. Summary of various groups and drugs administered during the 4-week treatment study.

| Group No. | Group name     | Drug and dose     |
|-----------|----------------|------------------|
| I         | NPD            | 3% v/v Tween-80  |
| II        | HFD (Control)  | 3% v/v Tween-80  |
| III       | HFD + Orlistat (reference) | Orlistat (10 mg/kg) |
| IV        | HFD + 11b (Low dose) | 11b (10 mg/kg)   |
| V         | HFD + 11b (High dose) | 11b (20 mg/kg)   |
and 40 ppm, while the -CH2 carbon attached to the nitrogen of the indole resonated at 45 ppm.

### 3.2. Pancreatic lipase inhibition assay and enzyme kinetics

All the synthesized analogues were evaluated for their PL inhibitory activity and the results are summarized in Table 2. The PL inhibitory potential of these analogues varied between 1.6 to 8 μM, with a majority of the analogues exhibiting potent PL inhibitory activity (IC\textsubscript{50} < 5 μM). The most potent activity was found with 11b with an IC\textsubscript{50} value of 1.68 μM, while comparable to the standard drug, orlistat (IC\textsubscript{50}/C\textsubscript{0} 0.99 μM). In addition, four more analogues, 9b, 9c, 10b and 11c, exhibited better potential with IC\textsubscript{50} values of 2.84, 2.61 and 2.24 μM, respectively (Figure S1, Supplementary Data), compared to analogue 5 (IC\textsubscript{50}/C\textsubscript{0} 2.95 μM). Further, enzyme inhibition kinetics of analogue 11b indicated its reversible competitive nature of inhibition. As represented in Figure 4 and Table 3, the plots in the Lineweaver-Burk plot converged at y-intercept (1/V\textsubscript{max}) while the Km values (slope of the plots) increased proportionally with inhibitor concentration. This fact is in line with our previous studies, wherein the indolyl oxoacetamides 4 and 5 also exhibited a reversible competitive inhibition of PL (Sridhar et al., 2019, 2020). Further, analogue 11b exhibited a K\textsubscript{i} value of 0.98 μM, as calculated using the Cheng-Prusoff equation (Burlingham & Widlanski, 2003).

### 3.3. Structure-activity relationship

A preliminary structure-activity relationship (SAR) analysis of the benzyloxy substituted indole glyoxylamides is provided in Figure 5. At R, 7-benzyloxy and 6-benzyloxy substitutions resulted in greater potency over the 5-benzyloxy counterpart, while the 4-benzyloxy substitution exhibited comparatively poor inhibition.

For the substitution at Ar, the 2-(1H-indol-3-yl)ethyl moiety resulted in a better potency over the trimethoxybenzyl substitution followed by the trimethoxyphenyl substitution. Nevertheless, the PL inhibitory activities of the respective counterparts were not found to be significantly different. Further, this relation was not observed with the 5-benzyloxy analogues, wherein the trimethoxybenzyl moiety resulted in lower potency over the other two substitutions at Ar.

### 3.4. Molecular docking analysis

For molecular docking studies, the energy minimized structures of the ligands were docked into the crystal structure of Human PL (PDB ID: 1LPB) using Molegro Virtual Docker 6.0. The MolDock scores of the analogues exhibited significant correlation to their PL inhibitory activity (Pearson’s r = 0.7122, p < 0.05), with the most potent analogue 11b exhibiting potential MolDock score of −186.456 kcal/mol. All the analogues exhibited π-π interactions with the amino acids of the lid domain viz., Phe77 and/or Phe215 (Figure 6), while also exhibiting extended alkyl interactions with the lid domain (Table 4). This, however, was not seen with the non-benzyloxy counterpart, 5, validating our hypothesis to include benzyloxy substitution to enhance the pancreatic lipase inhibitory potency (Table 4).

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Table 2. In vitro PL inhibitory activity of benzyloxy substituted indole glyoxylamides.

| # | R Ar | IC\textsubscript{50} (μM) | # | R Ar | IC\textsubscript{50} (μM) |
|---|---|---|---|---|---|
| 9a | 5-benzyloxy 3,4,5-trimethoxyphenyl | 3.89 ± 0.27 | 10c | 6-benzyloxy 3,4,5-trimethoxybenzyl | 3.03 ± 0.72 |
| 9b | 7-benzyloxy 3,4,5-trimethoxyphenyl | 2.84 ± 0.29 | 11a | 5-benzyloxy 2-(1H-indol-3-yl)ethyl | 4.44 ± 0.64 |
| 9c | 6-benzyloxy 3,4,5-trimethoxyphenyl | 2.61 ± 0.24 | 11b | 7-benzyloxy 2-(1H-indol-3-yl)ethyl | 1.68 ± 0.28 |
| 9d | 4-benzyloxy 3,4,5-trimethoxyphenyl | 7.91 ± 0.96 | 11c | 6-benzyloxy 2-(1H-indol-3-yl)ethyl | 2.24 ± 0.41 |
| 10a | 5-benzyloxy 3,4,5-trimethoxybenzyl | 5.22 ± 0.77 | 11d | 4-benzyloxy 2-(1H-indol-3-yl)ethyl | 7.73 ± 1.47 |
| 10b | 7-benzyloxy 3,4,5-trimethoxybenzyl | 2.34 ± 0.46 | 5 | -H | 2.95 ± 1.47 |

Figure 4. Double reciprocal Lineweaver-Burk plot of compound 11b obtained through enzyme kinetic study.

Table 3. V\textsubscript{max} and K\textsubscript{m} values calculated from Lineweaver-Burk plot at different concentrations of 11b [I].

| [I] (μM) | V\textsubscript{max} (μM.min\textsuperscript{-1}) | K\textsubscript{m} (μM) |
|---|---|---|
| 0 | 1.623 | 70.11 |
| 5 | 1.522 | 162.09 |
| 10 | 1.648 | 232.90 |
Figure 5. Structure-activity relationship for benzyloxy substituted indole glyoxylamides.

7-benzyloxy ≥ 6-benzyloxy > 5-benzyloxy > 4-benzyloxy

Figure 6. (A) 2D interaction diagram of 11b with PL; (B) 3D interaction diagram highlighting H-bond (green), π-π stacking (pink) and π-cation (brown) interactions of 11b with the active site of PL; (C) Representation of 11b in the binding pocket of PL, highlighting N-geranyl moiety in close proximity with lid domain (in brown).
The distance between the reactive carbonyl group and Ser152 also played a crucial role in PL inhibitory activity of these compounds. For instance, the poor PL inhibitory activity of the 4- and 5-benzyloxy analogues, as discussed under SAR, can be attributed to the greater distance between the reactive carbonyl and Ser152, while this distance was minimal with 6- and 7-benzyloxy substitutions (Figure 7). This fact is in line to our previous studies, wherein a greater distance between the reactive carbonyl group and Ser152 resulted in reduced PL inhibitory activity (Sridhar, Ginson, et al., 2017; Sridhar et al., 2019, 2020).

3.5. Molecular dynamics simulations

Molecular docking analysis clearly indicated the pivotal role of \( \pi-\pi \) stacking and alkyl interactions with the lid domain in imparting potent PL inhibitory activity. In an attempt to better understand the role of these interactions, analogue 11b was subjected to a 50 ns MD simulation in complex with PL. As represented in Figure 8(A), the backbone RMSD remained stable throughout the run with a maximum deviation of 4 Å, validating the MD simulation. Further, as represented in Figure 8(B), analogue 11b remained stable in the active site during the entire simulation. 11b exhibited an initial RMSD of 2 Å at 0 ns, and deviated up to 4 Å till 30 ns (Relative RMSD \( \approx 2 \) Å). After this, the RMSD reached to a maximum of 5 Å at 45 ns (Relative RMSD \( \approx 3 \) Å). Various interactions exhibited by 11b during the 50 ns MD run are represented in Figure 9 and Table 5. 11b has maintained stable H-bond interaction with Phe77, Tyr 114 and Phe215. Further, 11b also exhibited alkyl interactions with the amino acids of the lid domain viz., Phe77, Ile78, Leu213 and Phe215, alongside Arg256 and Ala259. In addition, 11b also maintained stable \( \pi-\)cation interaction with Arg256 during the entire 50 ns MD run (Table S2, Supplementary Data).

3.6. In silico prediction of gastro-intestinal absorption and toxicity

The results from the PL inhibition assay indicated three analogues, 10b, 11b and 11c, to possess potent activity (IC\(_{50}\) \( \leq 2.5 \mu M \)), highlighting their suitability for \textit{in vivo} experiments. Nevertheless, the \textit{in vivo} efficacy of a given drug candidate is predominantly affected, not only by its \textit{in vitro} profile, but also by the various pharmacokinetic parameters and the toxicity properties (Moroy et al., 2012). Hence, these analogues were predicted for their GI absorption and toxicity profiles, alongside analogue 5 and orlistat. As summarized in Table 6, analogue 5 possessed lower logP\(_{O/W}\) of 5.53 and high GI absorption. On the contrary, all the three analogues in the present study (10b, 11b and 11c) possessed a greater logP\(_{O/W}\) (6.09, 6.59 and 6.69) and low GI absorption comparable to that of the orlistat (logP\(_{O/W}\) = 7.05).

For the toxicity profile, various parameters viz, oral toxicity, carcinogenicity (genotoxicity, non-genotoxicity) and hepatotoxicity were predicted. As summarized in Table 6, orlistat possessed a predicted LD\(_{50}\) of 1300 mg/kg, and was also found to be genotoxic. In addition, orlistat was also predicted to be hepatotoxic, which is in line with the recent FDA reports (http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm213038.htm). On the contrary, analogues 10b, 11b, 11c as well as 5 were predicted to be non-carcinogenic and non-hepatotoxic in contrary to orlistat. However, the predicted LD\(_{50}\) values of 11c and 5 (with LD\(_{50}\) value of 1000 mg/kg) highlighted a greater risk of oral toxicity, while 10b and 11b possessed comparatively
greater LD<sub>50</sub> value (5000 mg/kg) indicating their suitability for the <i>in vivo</i> experiments. Consequently, analogue 11b was selected over 10b for the <i>in vivo</i> experiments owing to its greater PL inhibitory potency and log<sub>O/W</sub>.

### 3.7. In vivo experiments

For the <i>in vivo</i> experiments, male Swiss albino mice (15–20 g) were purchased from the Central Animal Facility of Birla Institute of Technology and Science, Pilani (BITS Pilani), Pilani Campus (India) (Registration number: 417/PO/ReBi/2001/CPCSEA) and treated according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). All the experimental procedures on animals were conducted in compliance with the Institutional Animal Ethics Committee of BITS Pilani (protocol No: IAEC/RES/20/06/Rev-2/24/20).

Briefly, three studies were conducted, namely (a) Oral Triglyceride Tolerance Test, (b) Long term (4-week) treatment study and (c) Quantification of faecal triglycerides.

#### 3.7.1. Oral Triglyceride Tolerance Test

The Oral Triglyceride Tolerance Test (OTTT) was conducted as per the previously reported literature to examine the effect of 11b on the intestinal absorption of triglycerides as well as to calculate its dose for the long term treatment study (T. Y. Chen et al., 2018). As represented in Figure 10(A), the triglyceride levels increased drastically to 197.217 and 161.842 mg/dL in the positive control group followed by the low dose (5 mg/kg) group of 11b (group 1 and 4, respectively) at 1.5 h. On the contrary, administration of medium and high dose of 11b (group 5 and 6, respectively) did not result in significant increase in the triglyceride levels. Moreover, the triglyceride levels in these groups were not significantly (<i>p</i> < 0.05) different from the orlistat group (group 3), calculated at similar time point. These results clearly indicated that 11b at 10 and 20 mg/kg dose exhibited profiles comparable to the standard drug orlistat (at 10 mg/kg), and hence were considered for long-term treatment study alongside orlistat (10 mg/kg).

#### 3.7.2. Long-term treatment study

For the long-term treatment study, various parameters including body weight, serum glucose and serum lipid profiles were estimated at the end of every week. As represented in Figure 10(B), 11b at 20 mg/kg exhibited comparable results as that of the orlistat treated group by the end of 4th week (34.87 ± 0.71 and 37.33 ± 0.65 g, respectively). Similarly, 11b at 20 mg/kg exhibited significantly comparable efficacy as that of orlistat in various biochemical parameters, with an exception for serum glucose and LDL-cholesterol.

As represented in Figure 11(B,C), the serum triglycerides and serum total cholesterol were found to be 94.95 and 94.40 mg/dL, respectively, for 11b (20 mg/kg) treated groups, while for orlistat (10 mg/kg), these values were found to be 83.85 and 90.96 mg/dL. However, the lower
Figure 8. Backbone RMSD (A) and ligand RMSD (B) retrieved from 10 ns MD simulation of 11b – PL complex.

Figure 9. 2D representation of various interactions exhibited by 11b with PL during the 50 ns MD simulation.

Table 5. Various interactions exhibited by 11b during the 50 ns MD run.

| Time frame (ns) | H-bond | π-π stacking | π-cation interactions | π-alkyl |
|----------------|--------|--------------|-----------------------|--------|
| 0              | Phe77  | Phe77, Phe215, Trp252, His263 | Arg256 | Ile78, Ala178, Arg256, Ala259 |
| 5              | Phe77  | Phe77, Phe215, Thr255 | –         | Ile78, Arg256, Ala259 |
| 10             | Phe77  | Tyr114, Phe215 | Arg256 | Phe77, Ile78, Ala178, Arg256, Ala259 |
| 15             | Phe77  | Phe77, Tyr114, Phe215 | Arg256 | Phe77, Ala178, Arg256, Ala259 |
| 20             | Phe77  | Phe77, Tyr114, Phe215 | –         | Phe77, Ile78, Ala178, Ile209, Arg256, Ala259 |
| 25             | Phe77  | Phe77, Tyr114, Phe215 | –         | Arg256, Ala259 |
| 30             | Phe77  | Phe77, Tyr114 | –         | Phe77, Ile78, Ala178, Ala259 |
| 35             | Phe77  | Phe77, Tyr114, Phe215 | –         | Phe77, Ile78, Ala178, Ala259 |
| 40             | Phe77  | Tyr114, Phe215 | Arg256 | Phe77, Ala259 |
| 45             | Phe77  | Phe77, Phe215 | –         | Phe77, Ile78 |
| 50             | Phe77  | Thr255 | –         | Phe77, Ile78, Arg256 |

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Table 6. LogP\textsubscript{O/W}, GI absorption and toxicity profiles of the most potent analogues.

| #    | PL inhibition | Consensus | GI Absorption | Toxicity          |
|------|---------------|-----------|---------------|-------------------|
|      | IC\textsubscript{50} (µM) | LogP\textsubscript{O/W} |                | LD50 | Genotoxic | Non genotoxic | Hepatotoxic |
| 10b  | 2.34 ± 0.46   | 6.09      | Low           | 5000 | No   | No          | No         |
| 11b  | 1.68 ± 0.28   | 6.59      | Low           | 5000 | No   | No          | No         |
| 11c  | 2.24 ± 0.41   | 6.69      | Low           | 1000 | No   | No          | No         |
| 5    | 2.95 ± 0.38   | 5.53      | High          | 1000 | No   | No          | No         |
| Orlistat | 0.99 ± 0.11 | 7.05      | Low           | 1300 | Yes  | No          | Yes        |

*LD50 was predicted in mg/kg and is an indicator for oral toxicity.*

Figure 10. (A) Graphical representation of results from the OTTT summarizing the serum triglyceride levels at various time points (n = 4; p < 0.05); (B) Increment in body weights of various groups during the four-week treatment protocol.

Figure 11. (A) Serum glucose levels (\textsubscript{\textasteriskcentered}p < 0.001 vs. NPD; \textsubscript{\textasteriskcentered}# p < 0.01 vs. HFD; \textsubscript{\textasteriskcentered}p vs. HFD); (B) Serum triglyceride and (C) Serum total cholesterol (\textsubscript{\textasteriskcentered}p < 0.001 vs. NPD; \textsubscript{\textasteriskcentered}ns vs. NPD); (D) Serum HDL-cholesterol (\textsubscript{\textasteriskcentered}p < 0.001 vs. NPD; \textsubscript{\textasteriskcentered}ns vs. NPD; \textsubscript{\textasteriskcentered}p < 0.001 vs. HFD + Orlistat; \textsubscript{\textasteriskcentered}p < 0.05 vs. HFD + Orlistat); (E) Serum LDL-cholesterol (\textsubscript{\textasteriskcentered}p < 0.001 Vs. NPD; \textsubscript{\textasteriskcentered}ns Vs. NPD; \textsubscript{\textasteriskcentered}p < 0.001 Vs. HFD + Orlistat) [All the biochemical parameters were determined after the 4-week treatment period and the values are represented as mean ± SEM; (F) Faecal triglyceride levels determined from various groups. (All the values are represented as mean ± SEM calculated from four readings, corresponding to four weeks. \textsubscript{\textasteriskcentered}p < 0.001 Vs. NPD; \textsubscript{\textasteriskcentered}# p < 0.01 Vs. NPD; \textsubscript{\textasteriskcentered}ns Vs. HFD; \textsubscript{\textasteriskcentered}ns Vs. HFD = Orlistat.)
dose of 11b (10 mg/kg) was found to be less effective, as it exhibited significant differences in all the biochemical parameters when compared to orlistat (10 mg/kg).

3.7.3. Quantification of faecal triglycerides
Since PL inhibition is characterized by the excretion of faeces rich in triglycerides, the faeces of the mice were collected daily during the long-term treatment period, and triglycerides were quantified at the end of every week. As presented in Figure 11(F), the faecal triglyceride levels were significantly higher in the HFD control compared to the NPD group (43.03 ± 2.89 mg/g vs. 20.93 ± 1.41 mg/g). Further, these levels increased significantly to 67.33 and 59.31 mg/g, respectively, with the administration of orlistat (10 mg/kg) and 11b (20 mg/kg), while the triglyceride levels in these two groups did not exhibit any significant difference, indicating 11b acted through PL inhibition similar to orlistat.

4. Conclusion
In conclusion, the present study resulted in the design and synthesis of novel benzoyloxy substituted indole glyoxylamides based on lead 5, wherein analogue 11b exhibited very potent and reversible competitive inhibition of PL (IC50 = 1.68 μM), comparable to the standard drug, orlistat (IC50 = 0.99 μM). The MolDock scores of these analogues were in good agreement with the in vitro activity (Pearson’s r = 0.7122, p < 0.05). Moreover, the results from the PL inhibition assay and molecular docking analysis highlighted C6 and C7 positions of the indole glyoxylamides as favourable for benzoyloxy substitutions. Prediction of GI absorption and toxicity profiles of the most potent analogues, 10b, 11b and 11c, highlighted analogue 11b as a suitable candidate for in vivo experiments. The results from in vivo experiments indicated that 11b at 20 mg/kg dose exhibited comparable pharmacological efficacy to orlistat (10 mg/kg), while faecal triglyceride quantification confirmed that analogue 11b acted through PL inhibition.

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Disclosure statement
The authors declare no conflicts of interest.

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