8. ADMET PREDICTION AND *IN-VIVO* EXPERIMENTS

Chapter VIII

8. ADMET Prediction and *In-vivo* Experiments

8.1. Rationale

Previous chapters have discussed the development of potent PL inhibitors *via* various structural modification approaches. The net desired pharmacological activities of drugs/ drug candidates are not only dependent on their *in-vitro* potential but also on the pharmacokinetic fate (*i.e* ADME). Generally, the drug discovery and development is a complicated and lengthy process that deals with the huge financial and time investments [1,2]. Due to the unintended side/adverse effects and lack of site delivery, numerous drug candidates fail during the drug discovery program. In order to reduce the attrition rate during the discovery process, the fate of drug candidates can be predicted by using numerous *in-silico* molecular and/or data modelling methods. During the molecular modelling methods, quantum mechanical methods are used, while in the data modelling methods the quantitative structure-activity relationship (QSAR) approach is utilised [3]. These approaches offer an option to reduce the overall attrition rate during the drug discovery process as well as the fastening of these process *via* focussing on the most active hit compounds produced by various structural modifications [4].

By considering the importance of these approaches in the drug discovery process, the present chapter mainly deals with the understanding of the ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) parameters of the most potential analogues from the previous chapters, followed by the *in-vivo* evaluation of the most active analogue.

8.2. Material and Methods

8.2.1. ADMET Predictions

A total of top 14 potent analogues were considered for ADMET predictions based on their *in-vitro* PL inhibitory activity along with the echitamine (natural product lead) and orlistat (positive control). Analogues with IC_{50} less than 10 μM (Series I & II) and 6 μM (Series III) were selected. Various online tools such as SwissADME, OSIRIS property explorer and GUSAR were used for the determination of the ADMET profiles [5–7].

8.2.2. *In-vivo* Experiments

8.2.2.1. Experimental protocol

All the experimental procedures on animals were in compliance with the Institutional Animal Ethics Committee of BITS Pilani (Ref No: IAEC/RES/28/16). Briefly, the protocol was divided into three phases.

i)Oral Triglyceride Tolerance Test
ii) Anti-obesity studies (4 weeks treatment)
iii) Quantification of faecal triglycerides

8.2.2.2. Animals and Diet

Male Swiss albino mice (15–20 g) were purchased from the Central Animal House of Birla Institute of Technology and Science, Pilani (BITS Pilani), Pilani Campus, India (CPCSEA Reg. Number: 417/PO/ReBi/2001/CPCSEA**)**. The mice were housed in polyacrylic cages and maintained under standard husbandry conditions (room temperature 22 ± 1°C and relative humidity of 60%) with a 12-h light/dark cycle.

i) Oral Triglyceride Tolerance Test (OTTT)

To examine the *in-vivo* effects on the intestinal absorption of triglycerides and rationalising the dose of **7r** for anti-obesity effect (4 week treatment regimen), OTTT was performed according to the previously reported protocol [8,9]. The mice were divided into six groups (n=6) and were deprived of food for 18 h before the experiment. The dose of orlistat was considered as 10 mg/kg. Low (5 mg/kg), medium (10 mg/kg) and high dose (20 mg/kg) of test analogue (**7r**) were evaluated. The mice were orally administered with (1) olive oil as a positive control, (2) filtered water as a negative control, (3) olive oil plus orlistat (10 mg/kg) as reference control (4) olive oil plus **7r** (Low, medium and high dose). The amount of olive oil administered per animal was fixed to 5 mL/kg. Blood samples were collected at 0, 1.5, 3, 4.5, and 6 h after administration of olive oil, and triglyceride contents were detected using commercially available kits (Spinreact S.A.U, Spain).

ii) Anti-obesity studies (4-week treatment)

The anti-obesity effect of **7r** on fat absorption was examined by administering **7r** to HFD fed mice over 4 weeks [10,11]. Five groups (**Table 8.1**) were considered, and each group included 7 animals (except for the control group, n=6). The animals were fed with either a normal pellet diet (NPD) or HFD and filtered water *ad libitum*. The activity of the **7r** was

monitored at 2 dose levels (medium & high). The dose of **7r** was decided based on the results of *in-vitro* PL inhibition assay results as well as OTTT.

Table 8.1 Summary of various groups and drugs administered for the *in-vivo* experiments.

#	Animal Group	Drug and Dose
1.	Normal pellet diet (NPD) control	-
2.	High-fat diet (HFD) Control	-
3.	HFD + Orlistat (10 mg/kg)	Orlistat (10 mg/kg)
4.	HFD + Low dose	7r (10 mg/kg)
5.	HFD + High dose	7r (20 mg/kg)

Prior to the treatment, all the animals under HFD groups were adapted to the HFD composition for a period of two weeks. The HFD used for the study is summarized in **Table 8.2**, and it contained 20% protein, 45% lipids and 35% carbohydrates by weight [12].

Table 8.2 Composition of the HFD used in the *in-vivo* experiments.

Ingredients	Qty (g/kg)	Ingredients	Qty (g/kg)
Casein	200	Lard	245
L-Cysteine	3	Soybean oil	25
Starch	125	Vitamin and mineral mix	53
Sucrose	72.8	NaCl	10
Cellulose	50	Cholic acid	2

After the adaptation period, **7r**/orlistat was dissolved in 0.3 %v/v CMC and administered to the animals using oral gavage.

The body weights of the animals were recorded weekly. The animals were subjected to an overnight fast at the end of the study, followed by the collection of blood samples using the retro-orbital puncture. The blood samples were centrifuged (1500 g) for obtaining the serum. Various biochemical parameters including glucose, total triglycerides (TG), total cholesterol (TC), and high-density lipoproteins-cholesterol (HDL) were estimated from the serum using commercially available diagnostic kits (Spinreact S.A.U., Spain and Accurex Biomedical Pvt. Ltd., India). The low-density lipoprotein-cholesterol (LDL) was calculated using the formula (**8.1**) reported in the literature [13].

LDL = [(TC - HDL)*0.9] - [TG*0.1] …… Formula 8.1

iii) Quantification of faecal triglycerides

Since the inhibition of PL is characterized by the excretion of faeces rich in triglycerides, the faeces of the mice were collected during the 4-week treatment period, and triglycerides were quantified. The procedure for quantification of faecal triglycerides was performed as per the literature report with minor modifications [8,14–16]. Briefly, 1g of faeces was taken in a separatory funnel and was subjected to vigorous shaking with 0.15 M NaCl. To this suspension, chloroform/methanol (4:1, % v/v) was added. The mixture was allowed to separate. The lower organic phase was collected, filtered and dried *in vacuo*. The obtained triglycerides were dissolved in 1 mL ethanol, and the quantity of triglycerides was estimated using a commercial kit (Spinreact S.A.U., Spain).

8.2.2.3. Statistical analyses

All the data were represented as mean ± S.E.M, 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.

8.3. Results and Discussions

8.3.1. ADMET Predictions

A total of 14 analogues were selected for the *in-silico* ADMET predictions *via* SwissADME, OSIRIS data explorer and GUSAR. The obtained results are summarised in **Table 8.3**. Orlistat was found to possess a low GI absorption and did not possess good blood-brain barrier (BBB) permeation properties. Nevertheless, analogues from the Series I and II exhibited a high GI absorption and low BBB permeation properties. However, analogues in Series III substituted with prenyl functionality revealed low GI absorption, while the remaining analogues were predicted to absorbs from the GI tract. Further, the majority of the synthesized analogues were found to be a good substrate for CYP2C19, CYP2C9 and CYP 3A4. All the screened analogues were found to be devoid of the mutagenicity and tumorigenicity properties. Orlistat and echitamine possessed LD_{50} values of 2935 and 2006 mg/Kg (rat oral toxicity), whilst the LD_{50} values of synthesized analogues were in the range of 1151-8762 mg/Kg. The higher or comparable LD_{50} values suggested the probable safety profiles of these analogues under the *in-vivo* conditions.

Table 8.3 Summary of ADMET parameters predicted for the potential analogues from each series along with echitamine and orlistat

Series	Molecule	Absorption	Distribution		Metabolism Inhibitor					Toxicity			
		GI absorption	BBB permeant	Pgp substrate	CYP 1A2	CYP 2C19	CYP 2C9	CYP 2D6	CYP 3A4	Rat Oral toxicity *LD_{50}	Mutagenicity	HERG Inhibition Type 1	Tumori genicity
NP Lead	Echitamine	High	No	Yes	No	No	No	No	No	2645	No	Weak	No
I	**5r**	**High**	**No**	**No**	**No**	**Yes**	**Yes**	**No**	**Yes**	**2006**	**No**	Weak	**No**
	5t	High	No	No	Yes	Yes	Yes	No	Yes	1448	No	Weak	No
II	**6d**	**High**	**No**	**No**	**No**	**Yes**	**Yes**	**No**	**Yes**	**1584**	**No**	Weak	**No**
	6e	High	No	No	No	Yes	Yes	No	Yes	1556	No	Weak	No
	6k	High	No	No	No	Yes	Yes	No	Yes	1382	No	Weak	No
III	7d	High	No	Yes	Yes	Yes	Yes	No	Yes	1151	No	Weak	No
	7i	Low	No	Yes	No	Yes	Yes	No	Yes	2982	No	Weak	No
	7m	High	No	No	No	Yes	Yes	No	Yes	2762	No	Weak	No
	7q	High	No	No	No	Yes	Yes	No	Yes	1410	No	Weak	No
	7r	**Low**	**No**	**Yes**	**Yes**	**Yes**	**Yes**	**No**	**Yes**	**1575**	**No**	Weak	**No**
	7s	Low	No	Yes	Yes	No	No	Yes	Yes	1508	No	Weak	No
	7t	Low	No	Yes	Yes	No	No	Yes	Yes	1268	No	Weak	No
Reference	**Orlistat**	**Low**	**No**	**Yes**	**No**	**No**	**Yes**	**No**	**No**	**2935**	**No**	Weak	No

*Predicted value represented as mg/kg; Most potent analogue from each series and orlistat highlighted in grey and bold.

Hence, based on the *in-silico* ADMET predictions as well as the *in-vitro* PL inhibitory potentials **7r** was selected for further *in-vivo* pharmacological evaluations.

8.3.2. *In-vivo* Experiments

8.3.2.1. Oral Triglyceride Tolerance Test (OTTT)

Effects of the **7r** on the serum triglyceride level after ingestion of the oil was evaluated in the OTTT and the result are summarised in **Fig. 8.1**. After the oil ingestion, a rapid increase in the serum triglyceride level was observed in the oil control groups. On contrary, administration of orlistat prevented the up shooting of the triglyceride levels, that indicated the potential towards the triglyceride metabolism. Nevertheless, the high and medium doses (20, 10 mg/Kg) of the **7r** exhibited a comparable result to that of orlistat, while the lower dose (5 mg/Kg) did not exerted a potential effect on the triglyceride absorptions.

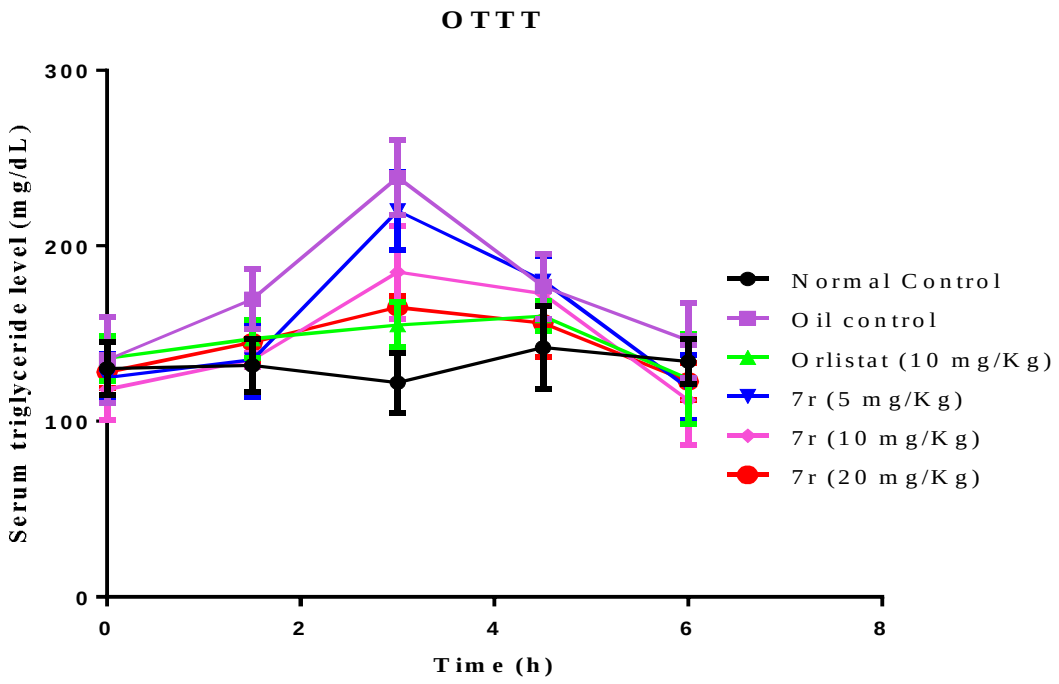

Fig. 8.1. Results of the OTTT summarising the serum triglyceride levels at various time points.

Based on the above results, high and medium doses (20, 10 mg/Kg) of the **7r** were selected for the evaluation anti-obesity effect *i.e.*, 4-week treatment study.

8.3.2.2. Anti-obesity studies (4 weeks treatment)

Anti-obesity effect of **7r** in the HFD feed mice was evaluated for four weeks. Two doses of **7r** (10, 20 mg/Kg) were selected, whilst orlistat (10 mg/Kg) was used as a reference control. The body weights of the animals were determined at the end of each week and the observed results are summarised in **Fig. 8.2.** The weight of the HFD group was significantly increased in comparison with the NPD control. However, the treatment groups revealed a significant reduction in body weight gain.

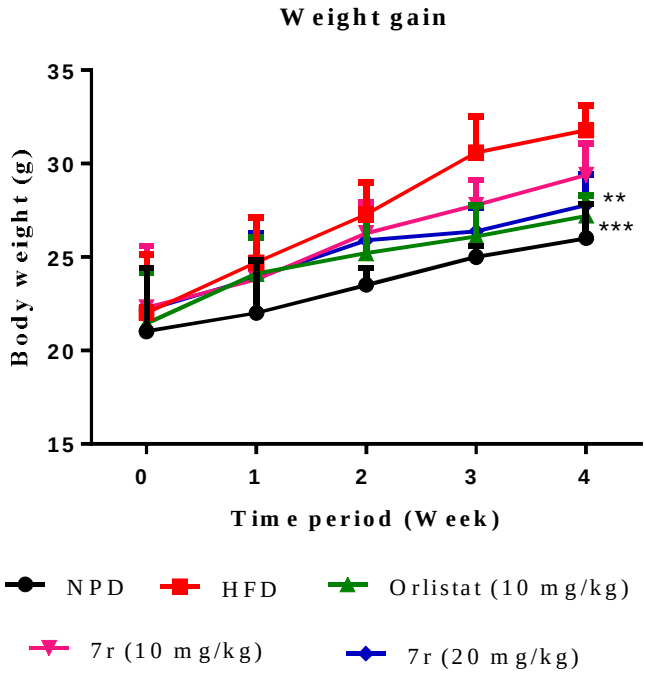

Fig. 8.2. Increment in body weights of various groups during the treatment period. The values are represented as mean ± SEM. ***$p \leq 0.001$, HFD *vs* orlistat; **$p \leq 0.01$, HFD *vs* **7r** (20 mg/Kg).

Apart from this, various biochemical parameters such as serum glucose, triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoproteins (LDL) were also measured and the obtained results are summarised in **Fig. 8.3.** Except for HDL, all the biochemical parameters of HFD control exerted significant changes to that of normal control. The orlistat treatment group resulted in the reversal of all the serum biochemical parameters. Both of the selected doses of **7r** resulted in the greater reversal of glucose profiles of the HFD mice. In the case of HDL and LDL, the low dose of **7r** did not exert significant changes compared to the HFD mice. Overall, these studies validated the anti-obesity potential of **7r** during the 4-week treatment.

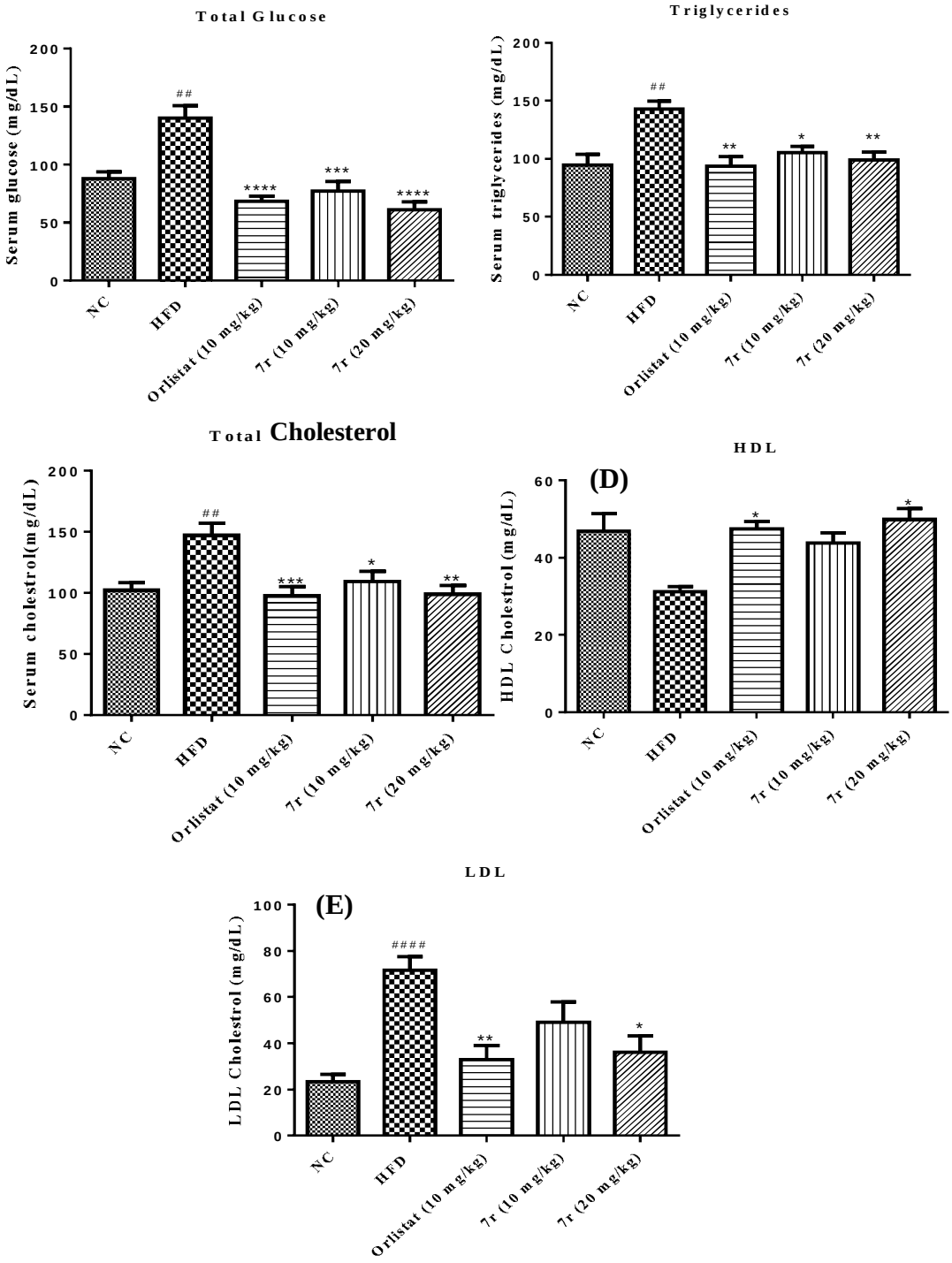

Fig. 8.3. Various biochemical parameters determined after the treatment period.

The values are represented as mean ± SEM. (**A**): $^{\#\#}$ $p \leq 0.01$, NC *vs* HFD; **** $p \leq 0.0001$, HFD *vs* orlistat and **7r** (20 mg/Kg); *** $p \leq 0.001$, HFD *vs* **7r** (10 mg/Kg). (**B**): $^{\#\#}$ $p \leq 0.01$, NC *vs* HFD; ** $p \leq 0.01$, HFD *vs* orlistat and **7r** (20 mg/Kg); * $p \leq 0.05$, HFD *vs* **7r** (10 mg/Kg). (**C**): $^{\#\#}$ $p \leq 0.01$, NC *vs* HFD; *** $p \leq 0.001$, HFD *vs* orlistat; ** $p \leq 0.01$, HFD *vs* **7r** (20 mg/Kg); * $p \leq 0.05$, HFD *vs* **7r** (10 mg/Kg). (**D**): * $p \leq 0.05$, HFD *vs* orlistat and **7r** (20

mg/Kg). (**E**): #### $p \leq 0.0001$, NC *vs* HFD; ** $p \leq 0.01$, HFD *vs* orlistat; * $p \leq 0.05$, HFD *vs* **7r** (20 mg/Kg).

8.3.2.3. Quantification of faecal triglycerides

From the previous studies, it is evident that the **7r** possessed a potential anti-obesity effect. OTTT suggested the potential of this analogue in the triglyceride absorption, while the various serum biochemical parameters suggested the overall anti-obesity potential. Since PL inhibition alters the digestion of the triglycerides, an excess amount of it is observed in the faeces. In order to evaluate the extent of anti-obesity effect, that was exerted through the PL inhibition, the amount of the triglycerides present in the faeces were quantified and the results are summarised in **Fig. 8.4**.

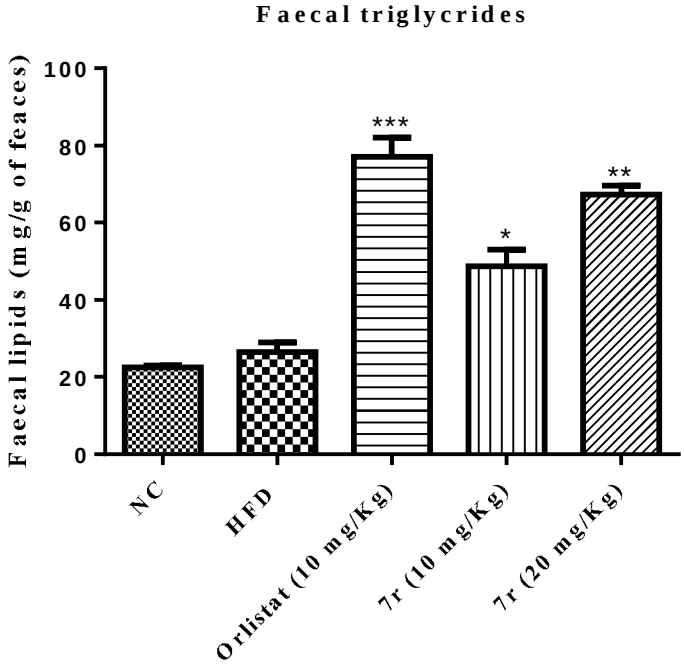

Fig. 8.4. Faecal triglyceride levels determined from various groups. The values are represented as mean ± SEM; (**C**): *** $p \leq 0.001$, HFD *vs* orlistat; ** $p \leq 0.01$, HFD *vs* **7r** (20 mg/Kg); * $p \leq 0.05$, HFD *vs* **7r** (10 mg/Kg).

The faecal triglyceride contents in the orlistat/ treatment groups significantly varied when compared to HFD/normal control groups. Amongst these groups, orlistat treatment group exhibited the maximum faecal triglyceride content followed by analogue **7r** (20 mg/Kg). Overall, quantification of the triglyceride contents revealed that the screened analogue (**7r**) had a prominent role in the fat absorption process, *via* inhibition of the PL.

In summary, the present chapter discussed the ADMET properties predictions of the synthesized analogues followed by the pharmacological evaluation of the topmost active analogues from all the 3 series. Incorporation of the isoprenyl functionality in the active pharmacophore resulted in the enhancement of the PL inhibitory potential. Further ADMET studies suggested that these analogues had lesser GI absorption properties, which was due to the enhancement of overall lipophilicity of these analogues. Further, these analogues possessed higher LD_{50} values, which further suggested the safety profiles of the analogues after the consumptions. All of the screened analogues were devoid of any kind of toxicity. Based on these results from the ADMET predictions and *in-vitro* PL inhibitory potentials, pharmacological evaluation of **7r** was evaluated in the *in-vivo* conditions. Three sets of experiments were performed for the proper understanding of the anti-obesity potential of the selected analogue (**7r**). OTTT studies indicated the role of **7r** in the triglyceride absorption, while four-week treatment studies in HFD feed mice suggested the anti-obesity potential. The faecal triglyceride study provided information about the PL inhibitory profiles of **7r** where it exerted an anti-obesity effect comparable to that of orlistat. Further, faecal triglyceride estimation clearly indicated that this anti-obesity effect was due to the inhibition of PL.

References

[1] F. Cheng, W. Li, G. Liu, Y. Tang, In Silico ADMET Prediction: Recent Advances, Current Challenges and Future Trends, Curr. Top. Med. Chem. 13 (2013) 1273–1289.

[2] S. Kar, J. Leszczynski, Open access in silico tools to predict the ADMET profiling of drug candidates, Expert Opin. Drug Discov. 15 (2020) 1473–1487.

[3] H. van de Waterbeemd, E. Gifford, ADMET in silico modelling: Towards prediction paradise?, Nat. Rev. Drug Discov. 2 (2003) 192–204.

[4] QikProp | Schrödinger. https://www.schrodinger.com/products/qikprop (accessed February 27, 2021).

[5] A. Daina, O. Michielin, V. Zoete, SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules, Sci. Rep. 7 (2017) 1–13.

[6] A. Lagunin, A. Zakharov, D. Filimonov, V. Poroikov, QSAR Modelling of Rat Acute Toxicity on the Basis of PASS Prediction, Mol. Inform. 30 (2011) 241–250.

[7] T. Sander, Molecular Properties Prediction - Osiris Property Explorer, Org. Cheistry Portal. (n.d.). https://www.organic-chemistry.org/prog/peo/ (accessed February 27, 2021).

[8] T.Y. Chen, M.M.C. Wang, S.K. Hsieh, M.H. Hsieh, W.Y. Chen, J.T.C. Tzen, Pancreatic lipase inhibition of strictinin isolated from Pu'er tea (*Cammelia sinensis*) and its anti-obesity effects in C57BL6 mice, J. Funct. Foods. 48 (2018) 1–8.

[9] S.A. Pai, E.A.F. Martis, S.G. Joshi, R.P. Munshi, A.R. Juvekar, Plumbagin exerts antiobesity effects through inhibition of pancreatic lipase and adipocyte differentiation, Phyther. Res. 32 (2018) 1631–1635.

[10] G. Avci, I. Küçükkurt, E. Küpeli Akkol, E. Yeşilada, Effects of escin mixture from the seeds of *Aesculus hippocastanum* on obesity in mice fed a high fat diet, Pharm. Biol. 48 (2010) 247–252.

[11] L.K. Han, Y. Kimura, M. Kawashima, T. Takaku, T. Taniyama, T. Hayashi, Y.N. Zheng, H. Okuda, Anti-obesity effects in rodents of dietary teasaponin, a lipase

inhibitor, Int. J. Obes. 25 (2001) 1459–1464.

[12] D.H. Pesta, V.T. Samuel, A high-protein diet for reducing body fat: Mechanisms and possible caveats, Nutr. Metab. 11 (2014) 1-8.

[13] Y. Chen, X. Zhang, B. Pan, X. Jin, H. Yao, B. Chen, Y. Zou, J. Ge, H. Chen, A modified formula for calculating low-density lipoprotein cholesterol values, Lipids Health Dis. 9 (2010) 1-5.

[14] K.A. Grove, S. Sae-Tan, M.J. Kennett, J.D. Lambert, (-)-Epigallocatechin-3-gallate inhibits pancreatic lipase and reduces body weight gain in high fat-fed obese mice, Obesity. 20 (2012) 2311–2313.

[15] T. Harach, O. Aprikian, I. Monnard, J. Moulin, M. Membrez, J.C. Béolor, T. Raab, K. MacÉ, C. Darimont, Rosemary (*Rosmarinus officinalis* L.) Leaf extract limits weight gain and liver steatosis in mice fed a high-fat diet, Planta Med. 76 (2010) 566–571.

[16] S. Uchiyama, Y. Taniguchi, A. Saka, A. Yoshida, H. Yajima, Prevention of diet-induced obesity by dietary black tea polyphenols extract *in-vitro* and *in-vivo*, Nutrition. 27 (2011) 287–292.