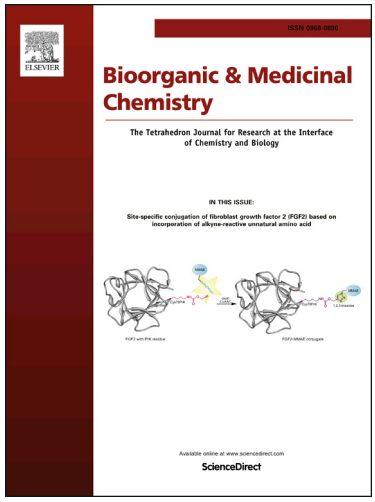
Accepted Manuscript



Synthesis, pH dependent, plasma and enzymatic stability of bergenin prodrugs for potential use against rheumatoid arthritis

Rohit Singh, Vikas Kumar, Sonali S. Bharate, Ram A. Vishwakarma

PII: S0968-0896(17)31330-5
DOI: http://dx.doi.org/10.1016/j.bmc.2017.08.011
Reference: BMC 13914

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 29 June 2017
Revised Date: 3 August 2017
Accepted Date: 8 August 2017

Please cite this article as: Singh, R., Kumar, V., Bharate, S.S., Vishwakarma, R.A., Synthesis, pH dependent, plasma and enzymatic stability of bergenin prodrugs for potential use against rheumatoid arthritis, *Bioorganic & Medicinal Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.bmc.2017.08.011

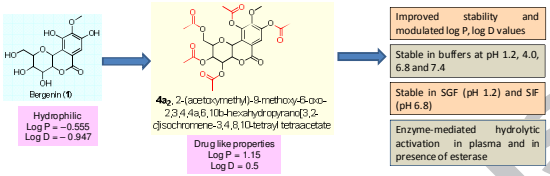
Graphical Abstract

Synthesis, pH dependent, plasma and enzymatic stability of bergenin prodrugs for potential use against rheumatoid arthritis

Leave this area blank for abstract info.

Rohit Singh[a,b], Vikas Kumar[a,b], Sonali S. Bharate[*a], and Ram A. Vishwakarma[*,b,c]

[a]Preformulation Laboratory, [b]Academy of Scientific and Innovative Research (AcSIR), [c]Medicinal Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India



ELSEVIER

Bioorganic & Medicinal Chemistry
journal homepage: www.elsevier.com

Synthesis, pH dependent, plasma and enzymatic stability of bergenin prodrugs for potential use against rheumatoid arthritis

Rohit Singh[a,b], Vikas Kumar[a,b], Sonali S. Bharate[*a], and Ram A. Vishwakarma[*,b,c]

[a]*Preformulation Laboratory,* [b]*Academy of Scientific and Innovative Research (AcSIR),* [c]*Medicinal Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India*

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Bergenin
Stability
Prodrugs
Lipophilicity
Enzymatic hydrolysis

ABSTRACT

Bergenin is a unique *C*-glycoside natural product possessing anti-inflammatory and anti-arthritic activity. It is hydrophilic molecule and stable under acidic conditions however is unstable at neutral-basic pH conditions. The rate of degradation is directly proportional to the increase in pH which might be one of the reasons for its low oral bioavailability. Thus, herein our objective was to improve its stability using prodrug strategy. Various ester and ether prodrugs were synthesized and studied for lipophilicity, chemical stability and enzymatic hydrolysis in plasma/esterase. The stability of synthesized prodrugs was evaluated in buffers at different pH, in biorelevant media such as SGF, SIF, rat plasma and in esterase enzyme. All prodrugs displayed significantly improved lipophilicity compared with bergenin, which was in accordance with the criteria of drug-like compounds. Acetyl ester **4a$_2$** appeared to be the most promising prodrug as it remained stable at gastric/intestinal pH and was completely transformed to the parent compound bergenin in plasma as desired for an ideal prodrug. The data presented herein, will help in designing stable prodrugs of unstable molecules with desired physicochemical properties in structurally similar chemotypes.

1. Introduction

Prodrugs are chemically oriented drug delivery tools wherein pharmacologically potent structures can be optimized so as to overcome its physicochemical, pharmaceutical and biopharmaceutical properties to make it more useful. The parent drug/s are modulated through a temporary chemical change through the covalent attachment of a chemical moiety (promoiety). The prodrug being a completely new chemical, it possesses a different physicochemical profile which might allow easier drug delivery. Prodrugs can be used for several reasons *viz.* enhancing solubility, permeability or chemical stability of parent compound. This may result in improving drug targeting, reduced side effects and reduced too rapid elimination of parent drug.[1] These are bioreversible derivatives of drug/s that undergo an enzymatic and/or chemical transformation *in vivo* to release the parent drug, which can exert the desired pharmacological effect.[2]

There are many literature reports wherein prodrugs have been utilized for improving stability of parent compound. Omeprazole, a proton pump inhibitor, is marketed as Prilosec® which has shown to reduce gastric acid secretion in animals and humans.[3] Omeprazole was not designed as a prodrug. However later it was found that it acts as a prodrug and is converted to active inhibitor of proton pump in the acidic environment per se. It undergoes chemical activation via molecular rearrangements in acidic environment to form the active sulfonamide.[4] To prevent this premature activation, delayed release capsules of omeprazole are available in the market for oral administration.[5] The formulation consists of enteric coated granules of omeprazole (10, 20 or 40 mg). It is formulated as enteric coated granules because it is acid labile and enteric coating protect it from pre-absorption degradation in acidic environment of stomach. Absorption begins when formulation leave the stomach i.e. 1-3 h after dosing (gastric emptying time).[6, 7] Azacitidine (Vidaza®) is investigational antineoplastic agent and considered as a potential inhibitor of nucleic acid biosynthesis. For the treatment of myelodysplastic syndromes, it is the first DNA hypomethylating agent approved by FDA. Azacitidine is administered by intravenous and subcutaneous route. It presents several drawbacks with respect to oral administration, such as sub-optimal physiochemical properties (hydrophilic nature: water solubility 89 mg/mL, Log P -3.1) and hydrolytic instability.[8] In this direction, Xu and coworkers prepared amino acid ester prodrugs of azacitidine to overcome its hydrolytic instability.[9] The aqueous solution of azacitidine readily gets hydrolyzed however its bisulfite prodrug is stable to such degradation at acidic pH. The prodrug also gets converted to active drug at physiological pH.[10] NSC-743380 (oncrasin-72) is an anticancer agent, was found to be unstable in injectable formulations.

*Corresponding authors. Tel.: +91-191-2585006; Fax: +91-191-2586333
E-mail addresses: sonalibharate@gmail.com (SSB); ram@iiim.ac.in (RAV)

Oncrasin-72 tends to form dimers in aqueous solutions resulting in its loss of activity, especially at low-pH conditions. Wu et al. reported oncrasin-266, a stable prodrug of oncrasin-72 with cyclohexylacetic acid as a promoiety.[11] The shelf-life of cefamandole, a second generation broad spectrum cephalosporin antibiotic, was improved by formation of nafate ester. Cycloserine (an antibiotics used to treat tuberculosis) under acidic conditions hydrolyses to hydroxylamine and D-serine. Merck chemist Norm Jensen discovered stable prodrug of cycloserine, pentizidone.[12] To improve metabolic stability of compounds, researchers have also attempted ether prodrugs.[13, 14] There are many reports of ether prodrugs which were prepared in order to improve lipophilicity which will eventually improve permeability of parent compound.[13] Japanese company Yamasa cooperation prepared ether prodrugs of antiviral drug; BV-araU with an objective to improve its stability in GI tract upon ingestion.[14] This illustrates utility of prodrugs in improving stability of parent compound/ drug candidates so as to enhance drug delivery and thus oral bioavailability.

Bergenin (**1**) is a *C*-glycoside of 4-O-methylgallic acid which naturally occurs in several plant genera like *Bergenia ciliata* and *Bergenia ligulata,*[15, 16] *Bergenia stracheyi* (rhizomes),[17] *Astilbe chinensis* (rhizomes),[18] *Dryobalanops aromatic* (stem bark),[19] *Ardisia elliptica* (stem bark)[20] and *Mallotus japonicas* (stem bark).[21] Bergenin (**1**) exhibits various pharmacological activities like anti-arthritic,[22] anti-inflammatory,[15] antitussive,[23] hypolipidemic,[24, 25] anti-arrhythmic,[26] hepatoprotective,[21] neuroprotective,[27] anti-fungal[28] etc. Recently, we reported that bergenin possess selective inhibition of IL-6 cytokine (inhibition of IL-6, IC_{50} = 208 nM; TNF-α, IC_{50} = 12.5 µM) both in in-vitro as well as in-vivo assays. It also displayed promising anti-arthritic activity in collagen-induced arthritis model.[15] The overview of various pharmacological activities of bergenin is shown in Figure 1.

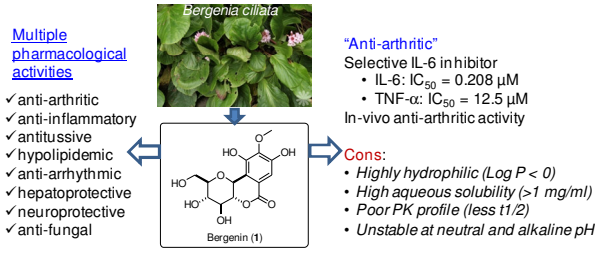


Figure 1. Pharmacological activities and physicochemical properties of bergenin

Literature reports indicate that bergenin gets degraded in neutral and alkaline conditions.[29] However, the drug was reported to be stable at acidic conditions at pH 1.0, 3.0 and 5.0. This indicate that bergenin is susceptible to degradation under neutral-basic pH conditions and the rate of degradation increases with increase in pH.[30] The degradation mechanism of bergenin indicates that the lactone bond gets hydrolyzed. The reason for low oral bioavailability of bergenin could be instability in neutral and alkaline pH such as intestinal tract.[31, 32] The physicochemical properties of bergenin are also reported by Zhou and coworkers.[30] They have reported the aqueous solubility of bergenin at different pH conditions *viz.* pH 1.0 (1.29 mg/mL), 3.0 (1.08 mg/mL) and 5.0 (1.22 mg/mL). The log P is -1.06 which clearly indicate the hydrophilic nature of bergenin. A Research group from China[29] has reported novel coated floating tablets of bergenin in combination with cetirizine dihydrochloride. The advantage of floating formulation is to prolong retention of bergenin in stomach thus avoiding its intestinal degradation which will in turn help to enhance its absorption and efficacy. However attempts have never been made to improve the stability of bergenin by prodrug approach. Herein, we report for the first time, the design and synthesis of prodrugs of bergenin in order to improve its stability and drug-like properties (Figure 2a and 2b). Ester and ether prodrugs of bergenin were synthesized using different promoieties. All synthesized prodrugs were evaluated for its stability at different pH conditions in order to identify an appropriate promoieties which will give stable prodrug/s with balanced lipophilicity. The stability of synthesized prodrugs was determined at different pH conditions and in biorelevant media *viz.* buffer solutions at pH 1.2, 4.0, PBS (pH 6.8), 7.4, SGF (pH 1.2), SIF (pH 6.8), rat plasma and in esterase enzyme from porcine liver. The experimental log P (water/octanol) and log D (PBS pH 7.4/ octanol) were determined by miniaturized shake flask method.

2. Results and discussion

2.1. Synthesis of ester and ether prodrugs of bergenin

Bergenin is a unique C-glycoside natural product present in different species of Bergenia. It is one of a major component of Bergenia ciliata plant. The repeated silica gel column chromatography of methanolic extract of this plant resulted in isolation of bergenin in grams. The isolated bergenin was characterized by detailed spectral analysis including NMR, MS, IR and 2D NMR analysis.

Designing ester and ether prodrugs is one approach to improve stability of bergenin having hydroxyl groups and modify/alter its physicochemical properties such as lipophilicity. It has been reported that ester prodrugs of a compound have been prepared to improve its stability and is an effective way to optimize drug-like properties of any compound. The esterases present in the body fluids are responsible for reversible conversion of prodrug to the parent form.[33, 34]

Higher alkyl chain fatty acids like hexanoic acid chloride, **2f**, have been utilized to generate lipophilic prodrugs such as haloperidol decanoate, a prodrug of haloperidol.[35] It is also reported that increase in degree of branching or length of the acyl side chain, **2b** and **2e**, will result in lipophilic prodrugs. Total 19 ester and ether prodrugs of bergenin were synthesized as depicted in Figure 2. All 19 compounds were then studied for Log P and Log D determination and investigated for their chemical and enzymatic stability. Bergenin on treatment with various acyl chlorides and alkyl halides resulted in formation of bergenin-esters and bergenin-ethers, respectively. All prepared prodrugs were characterized by NMR, MS, IR and HRMS studies. In ^{1}H NMR study, the presence of additional groups of acyl moiety confirmed the formation of acyl prodrugs. These observations were further confirmed by ^{13}C NMR and MS analysis. The position of acylation or etherification was confirmed by HMBC analysis, for which we selected four compounds viz. penta-substituted ester **4d$_1$**, mono-acyl product **4i$_1$**, diacyl product **4i$_2$** and di-substituted ether **5b**. Based on the observed HMBC correlations (2J and 3J) with adjacent carbons, the positions of acyl or alkyl groups was assigned. It was observed that etherification was primarily favored on aromatic hydroxyls e.g. all di-subsubtituted ethers were formed on two aromatic OH groups. However, all di-substituted esters were formed on one aromatic OH and one –CH_2OH of sugar moiety. This has been depicted in Figure 2 and HMBC data is presented in supporting information.

2.2. Determination of partition coefficient (log P) and distribution coefficient (log D)

Assessment of log P and log D is associated with physicochemical and physiological properties of the compound such as its membrane permeability.[36] Compounds with lower log P (< 0) are more polar and have poor membrane permeability however the compounds with higher log P (> 3) are more non-polar and have poor aqueous solubility. Log P and log D of synthesized esters (**4a-k**) and ethers (**5a-d**) was determined. We also determined the effect of different substituent (-R groups, aliphatic and aromatic) on partition coefficient and distribution coefficient of bergenin ester and ether prodrugs (Table 1). The log P and log D values followed opposite trend wherein the synthesized prodrugs **4a-k**, **5a-d** were hydrophobic (log P and log D in the range of 0.5 to 6.4) as compared to bergenin (log P, −0.5 and log D, −0.9). Aliphatic chain length was directly proportional to log P and log D value of bergenin prodrugs. Compounds **4e**, **4f** and **4j** had log P/D value of >5.0 indicating their hydrophobic nature and make them to deviate from the criteria for drug-like properties. Based on log P and log D values of bergenin prodrugs, compounds except **4a$_1$** (log P, −0.5 and log D, −1.0), **4e** (log P, 6.3 and log D, 6.4), **4f** (log P, 5.9 and log D, 6.0), **4j** (log P, 6.0 and log D, 6.1) and **4k** (log P, 3.7 and log D, 3.9) possess optimal log P and log D values (0-3).

2.3. In-vitro stability studies

It was observed that bergenin was unstable in PBS pH 6.8 (53% degraded in 24 h) and PBS pH 7.4 (70% degraded in 24 h). The hydrolytic study of synthesized ester and ether prodrugs of bergenin (**4a-k**, **5a-d**) initiated by determining compound's stability in PBS pH 7.4 buffer. The compounds which were found to be stable in PBS pH 7.4 were taken forward to determine their stability in different buffer solutions and in biorelevant media. These compounds were evaluated for stability towards hydrolysis in different media: 1) Buffer solutions of different pH *viz.* 1.2, 4.0 and 6.8 to examine their chemical hydrolysis, 2) SGF (pH 1.2) and SIF (pH 6.8) to evaluate their hydrolysis in biorelevant media, and 3) Rat plasma and esterase from porcine liver to understand enzymatic hydrolysis of synthesized prodrugs.

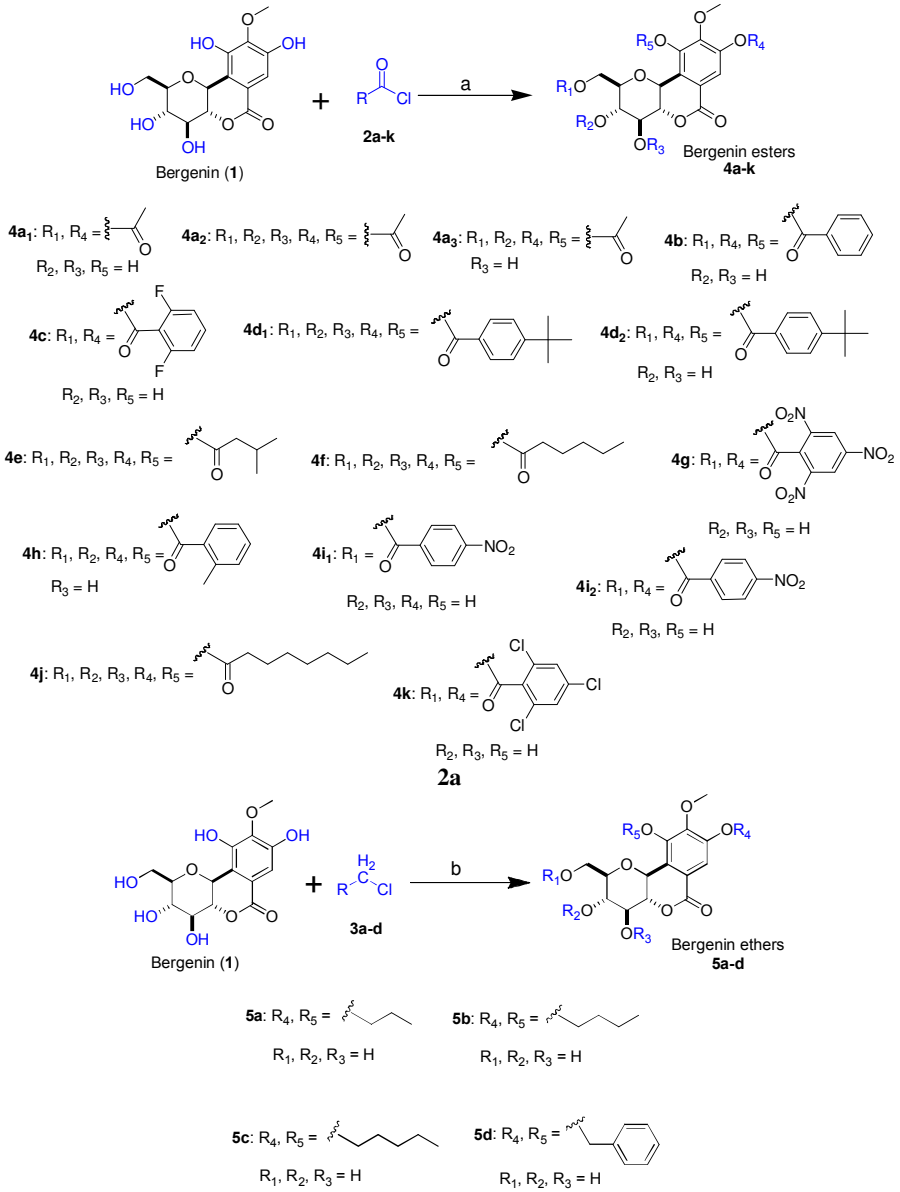


Figure 2. Synthesis of ester (**4a-k**) and ether (**5a-d**) prodrugs of bergenin (2a). Reagents and conditions: (a) Pyridine, 0-5 °C, 60–80 %. Acid chlorides *viz.* 2c, 2e, 2f, 2i, 2j and 2k were synthesized using thionyl chloride with 2,6-difluorobenzoic acid, 3-methylbutanoic acid, hexanoic acid, p-nitrobenzoic acid and octanoic acid respectively. (2b). Reagents and conditions: (b) KOH, DMF, RT with alkyl halide, 60–80 %.

Table 1
Experimental log P, log D and stability in PBS (pH 7.4) of bergenin and its prodrugs[a]

Compound	Partition coefficient at 25 °C	Distribution coefficient at 25 °C	% remaining after 24 h at 37 °C
	Log P (water/ n-octanol)	Log D (PBS pH 7.4/ n-octanol)	PBS (pH 7.4)
1	-0.555 ± 0.003	-0.947 ± 0.045	33.786 ± 3.277
4a$_1$	-0.494 ± 0.008	-1.062 ± 0.102	41.123 ± 6.877
4a$_2$	1.155 ± 0.035	0.516 ± 0.048	93.808 ± 10.227
4a$_3$	1.181 ± 0.020	0.429 ± 0.055	96.450 ± 2.619
4b	2.553 ± 0.175	1.773 ± 0.089	5.021 ± 1.617
4c	1.801 ± 0.072	1.861 ± 0.027	56.818 ± 20.375
4d$_1$	0.771 ± 0.055	0.162 ± 0.019	85.268 ± 11.647
4d$_2$	0.599 ± 0.252	0.552 ± 0.008	81.364 ± 12.738
4e	6.358 ± 0.036	6.453 ± 0.025	4.46 ± 0.830
4f	5.967 ± 0.025	6.064 ± 0.008	3.423 ± 1.455
4g	2.787 ± 0.104	1.798 ± 0.008	32.275 ± 10.328
4h	1.751 ± 0.179	1.608 ± 0.092	17.985 ± 3.552
4i$_1$	1.563 ± 0.172	1.535 ± 0.061	53.427 ± 7.952
4i$_2$	2.508 ± 0.146	2.655 ± 0.326	73.591 ± 9.881
4j	6.011 ± 0.028	6.113 ± 0.008	14.996 ± 4.857
4k	3.693 ± 0.155	3.888 ± 0.206	89.181 ± 21.547
5a	1.655 ± 0.019	1.508 ± 0.029	37.412 ± 13.289
5b	2.495 ± 0.032	2.835 ± 0.059	31.041 ± 11.29
5c	1.633 ± 0.113	1.524 ± 0.018	24.662 ± 5.469
5d	1.965 ± 0.184	1.981 ± 0.014	91.055 ± 17.344

[a], all values are average of three determinations and are represented as mean ± SD; nd, not determined

2.3.1. Chemical (non-enzymatic) hydrolysis in PBS pH 7.4

The % hydrolyzed after incubation of compounds in PBS pH 7.4 buffer for 24 h was determined and the values are shown in Table 2. The obtained results indicated that prodrugs *viz.* **4a$_2$**, **4a$_3$**, **4d$_1$**, **4d$_2$**, **4i$_2$**, **4k** and **5d** were stable to hydrolysis in PBS pH 7.4 buffer. The results are shown in Table 1, where % remaining after 24 h at 37 °C was found to be 93.8, 96.5, 85.3, 81.4, 73.6, 89.2 and 91.1% for **4a$_2$**, **4a$_3$**, **4d$_1$**, **4d$_2$**, **4i$_2$**, **4k** and **5d**, respectively. Therefore only these compounds were considered further to determine its stability at different pH and in different media. However, in case of bergenin, only 30% remained stable over a period of 24 h at 37 °C.

2.3.2. Chemical (non-enzymatic) hydrolysis in different buffers at pH 1.2, 4.0 and 6.8

The % hydrolyzed after incubation of compounds (**4a$_2$**, **4a$_3$**, **4d$_1$**, **4d$_2$**, **4i$_2$**, **4k** and **5d**) for 24 h in buffer solutions at pH 1.2, 4.0 and 6.8 was determined and the values are shown in Table 2. In HCl buffer at pH 1.2, compounds **4i$_2$**, **4k** and **5d** were degraded to the extent of 60.61, 23.6 and 49.2% respectively. It was also observed that **4i$_2$**, **4k** and **5d** were unstable in phosphate buffer pH 4.0 and degree of hydrolysis was 54.9, 24.1 and 47.6 respectively.

However, in PBS pH 6.8 buffer solution, compounds **4a$_2$**, **4a$_3$**, **4d$_1$**, **4d$_2$** and **4k** were stable with only 4-8% compound liable to degradation over a period of 24 h at 37 °C. **4i$_2$** and **5d** were susceptible to degradation with 48.2 and 53.4% remaining after 24 h. The compounds *viz.* **4a$_2$**, **4a$_3$**, **4d$_1$** and **4d$_2$** were stable to chemical hydrolysis at pH 1.2, 4.0 and 6.8 over 24 h at 37 °C.

2.3.3. Stability in biorelevant media, SGF (pH 1.2) and SIF (pH 6.8)

In vitro enzymatic hydrolysis pattern of ester prodrugs was studied in SGF (pH 1.2) and SIF (pH 6.8) at 37 °C. To mimic *in vivo* gastric and intestinal digestion, simulated gastric fluid with pepsin and simulated intestinal fluid with pancreatin were used for *in vitro* studies. The % hydrolysis of the compounds is depicted in Table 2. It was observed that prodrugs **4a$_2$**, **4a$_3$**, **4d$_1$**, **4d$_2$**, **4i$_2$** and **4k** were stable to hydrolysis in SGF pH 1.2. However, **5d** was hydrolyzed upto 31.2% after 24 h. When these stock solutions were incubated in SIF pH 6.8, compound **4a$_2$**, **4d$_1$**, **4d$_2$**, **4k** and **5d** were stable with >85% remaining after 24 h. In brief, only compounds **4a$_2$**, **4d$_1$**, **4d$_2$** and **4k** were stable to chemical hydrolysis and in biorelevent media namely SGF (pH 1.2) and SIF (pH 6.8). These four compounds were further selected to study their stability in rat plasma.

Table 2
Stability of selected bergenin prodrugs: chemical (non-enzymatic) and enzymatic hydrolysis

Compound	% remaining after 24 h at 37 °C					% remaining after 4 h at 37 °C
	HCl buffer pH 1.2	Phosphate buffer pH 4.0	PBS pH 6.8	SGF (pH 1.2)	SIF (pH 6.8)	Rat plasma
1	99.734 ± 10.21	102.431 ± 4.37	53.768 ± 3.46	111.045 ± 3.60	100.96 ± 14.16	82.584 ± 6.27
4a$_2$	109.05 ± 2.60	118.98 ± 3.72	93.339 ± 1.96	99.093 ± 3.56	100.123 ± 0.34	4.103 ± 0.48
4a$_3$	111.635 ± 2.23	122.03 ± 13.44	94.584 ± 15.96	92.321 ± 19.86	41.834 ± 4.024	ND
4d$_1$	99.95 ± 0.83	103.7 ± 7.02	93.251 ± 5.18	108.698 ± 7.01	86.083 ± 7.80	96.771 ± 6.49
4d$_2$	98.356 ± 0.58	95.041 ± 0.96	98.13 ± 3.67	105.541 ± 3.94	93.497 ± 2.42	92.951 ± 13.34
4i$_2$	40.39 ± 7.70	45.02 ± 6.27	51.775 ± 3.75	91.567 ± 7.64	59.553 ± 6.68	ND
4k	76.362 ± 0.37	75.99 ± 7.84	97.266 ± 5.78	86.456 ± 5.17	87.484 ± 4.35	86.5 ± 11.43
5d	50.862 ± 7.83	52.414 ± 2.35	46.60 ± 12.297	68.845 ± 9.23	90.529 ± 5.04	ND

2.3.4. Stability in rat plasma and esterase from porcine liver (PLE)

In vitro enzymatic hydrolysis of **4a$_2$**, **4d$_1$**, **4d$_2$** and **4k** was studied in rat plasma and in esterase from porcine liver at 37 °C. Compounds **4d$_1$**, **4d$_2$** and **4k** were stable to hydrolysis in rat plasma. The reason might be prodrugs (**4d$_1$**, **4d$_2$** and **4k**) prepared using substituted benzoyl chlorides as promoieties are less prone to degradation in comparison to prodrugs synthesized using acyl chloride (**4a$_2$**). The hydrolysis rate of **4a$_2$** in rat plasma and esterase was faster than the corresponding rate in HCl buffer (pH 1.2), phosphate buffer (pH 4.0), PBS (pH 7.4), SGF (pH 1.2) and SIF (pH 6.8). This indicated that the hydrolysis of **4a$_2$** is facilitated by enzymes and it was completely converted to parent compound bergenin in rat plasma and in esterase from porcine liver within 4 h (Table 2). The time dependent hydrolysis of **4a$_2$** in rat plasma and in esterase enzyme showed that 69% and 85% of **4a$_2$**, respectively, was converted immediately to bergenin upon mixing.

Briefly, the compound **4a$_2$** was found to have ideal properties that are required for any prodrug. Bergenin, being highly polar and unstable at intestinal pH, may be limiting factor for its absorption. Optimal lipophilicity is required for efficient absorption because most of the drugs are absorbed by passive diffusion. Thus, the prodrug **4a$_2$** with improved lipophilicity in comparison with bergenin, may promote its gastrointestinal absorption. Furthermore, the compound was very stable to chemical hydrolysis in buffer solutions of different pH and in SGF (pH 1.2) and SIF (pH 6.8). However, it was completely converted to parent compound i.e. bergenin in rat plasma and in presence of esterase from porcine liver in 15 min.

3. Conclusion

The stability of bergenin was improved successfully via prodrug approach. In total, 19 ester and ether prodrugs of bergenin were synthesized and investigated for their stability at different pH, log P and log D values. The data obtained in this study demonstrated that ester prodrugs of bergenin possess better lipophilicity, in comparison to bergenin which was in accordance with the criteria that is required in drug discovery. Log P and log D of all ester and ether prodrugs was found to be in the range of 0-3 which is as per the criteria required for the compounds in drug discovery. The best identified prodrug **4a$_2$** was stable to hydrolysis under different pH conditions. This was also substrate for the enzymes in plasma and other esterases leading to its conversion to the parent compound, bergenin. The selection of appropriate ester moiety was essential to control the conversion of ester to free bergenin in the presence of esterase enzymes. Preventing hydrolysis of ester prodrug in SGF (pH 1.2) and SIF (pH 6.8) was necessary to retain the molecule sufficiently lipophilic and in stable form to achieve intestinal absorption. Thus, stable and lipophilic prodrugs of compounds containing hydroxyl group may have potential to improve the transport of the prodrugs.

4. Experimental

4.1. General

Sodium dihydrogen phosphate, potassium phosphate monobasic, sodium acetate, boric acid, disodium hydrogen phosphate (Alfa Aesar), sodium chloride (Loba Chemie Pvt. Ltd.), pepsin, porcine stomach (Sigma-Aldrich), hydrochloric acid (SD Fine Chemicals), monobasic potassium phosphate (Alfa Aesar), pancreatin, porcine pancreas (Sigma-Aldrich), potassium chloride (Ranbaxy laboratories Ltd.), and sodium hydroxide (Qualigens Fine Chemicals) were used for preparation of buffers of different pH and biorelevant media for the study. HPLC grade methanol and acetonitrile (SD Fine Chemicals, Mumbai) was used throughout the study. n-Octanol (Sigma Aldrich) was used for partition and distribution coefficient experiments.

HPLC (Shimadzu, LC-6AD), reversed-phase C18 column (NeoSphere, 5 µm, 250 mm × 4.6 mm), reversed-phase C8 column (Discovery HS, 5 µm, 250 mm × 4.6 mm), vortex (IKA vortex Genius 3), microplate shaker (Grant-bio PMS-1000 Microplate Shaker, Digital), pH meter (Thermo electron co-operation orion 20A+), microcentrifuge 5430R (Eppendorf), sonicator and micropipettes were used for the study.

^{1}H, ^{13}C and DEPT NMR spectra were recorded on Brucker-Avance DPX FT-NMR 500 and 400 MHz instruments. ^{13}C NMR spectra were recorded at 125 MHz or 100 MHz. ESI-MS spectra were recorded on Agilent 1100 LC-Q-TOF machine. IR spectra and melting points were recorded on Perkin-Elmer IR spectrophotometer and digital melting point apparatus, respectively.

4.2. Isolation and characterization of bergenin (1)

The air-dried and powdered plant material of *Bergenia ciliata* (1.5 kg) was extracted with methanol using maceration method. Evaporation of the solvent produced 300 g of methanolic extract. MeOH extract was loaded on the silica gel (# 100-200) column chromatography and column was eluted with EtOAc: hexane followed by MeOH: $CHCl_3$, which resulted in isolation of bergenin (**1**, 12 g). White crystalline powder; HPLC purity: 98.8% (t_R = 5.68 min); m.p. 237-240 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.74 (s, 1H), 8.39 (s, 1H), 6.92 (s, 1H), 5.60 (d, *J* =4 Hz, 1H), 5.39 (d, *J*= 4 Hz, 1H), 4.92 (d, *J*= 12 Hz, 1H), 3.95 (t, *J*= 8 Hz, 2H), 3.80 (d, *J*= 16 Hz, 1H), 3.71 (s, 3H), 3.61-3.48 (m, 4H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 165.25, 152.82, 149.92, 142.41, 119.92, 117.79, 111.29, 83.59, 81.62, 75.51, 73.92, 72.72, 62.94, 61.67; IR ($CHCl_3$) ν_{max} 3585, 3370, 2922, 2851, 2346, 1653, 1422, 1154, 1021 cm^{-1}; ESI-MS: *m/z* 329.00 $[M+H]^+$; HRMS: *m/z* 329.0865 calcd for $C_{14}H_{16}O_9+H^+$ (329.0867).

4.3. Synthesis and purification of ester (4a-k) and ether (5a-d) prodrugs of bergenin

Bergenin (328 mg, 1.0 mM, 1 Eq) was dissolved in pyridine and the mixture was stirred for 10 min in an ice bath. To this solution, the respective acid chloride (0.5 Eq/OH) was added drop-wise under stirring. The reaction mixture was stirred at RT for 3 h and quenched by addition of cold water. The mixture was washed 3 times with saturated solution of $NaHCO_3$ followed by washing 3 times with brine. The resulting mixture was partitioned with ethyl acetate. The EtOAc layer was concentrated over vacuo rotavapor and the crude products were purified over silica gel column chromatography using EtOAc: Hexane as mobile phase to get ester products **4a-k** (Figure 2a).

Bergenin (328 mg, 1.0 mM, 1 Eq) was dissolved in DMF. The resulting mixture was stirred for 10 min at RT. To this solution, the respective alkyl halide (1 Eq) was added drop-wise under stirring and the reaction mixture was further stirred at RT for 3-4 h. The reaction was quenched by addition of cold water and washed 3 times with $NaHCO_3$ followed by brine, respectively. The resulting mixture was partitioned with ethyl acetate and this

layer was concentrated to get desired ether prodrugs **5a-d** (Figure 2b).

HPLC purity of compounds was carried out using RP-C18 column (NeoSphere, 250 mm × 4.6 mm, 5 µm). Mobile phase consisted of ACN: water (15:85 v/v) at flow rate of 1.0 mL/min (pump, Shimadzu LC-6AD). The column oven (CTO-10ASVP) temperature was 37 °C and the injection volume (SIL-20A HT Prominence autosampler) was 15 µL. The analysis was performed using diode array detector (SPD-M20A, Prominence, Shimadzu).

4.3.1. (8-Acetoxy-3,4,10-trihydroxy-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromen-2-yl)methyl acetate (***4a₁***): Pale yellow powder; HPLC purity: 99.6% (t_R = 2.35 min.); m.p. 220-223 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.65 (s, 1H), 5.66 (d, *J*= 4 Hz, 1H), 5.38 (d, *J*= 4 Hz, 1H), 4.74 (s, 1H, OH), 4.06 (t, *J*= 8 Hz, 2H), 3.82 (s, 3H), 3.67-3.64 (m, 2H), 3.44-3.33 (m, 2H), 3.15-3.14 (m, 1H), 2.39 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 170.51, 170.03, 164.33, 151.09, 144.96, 142.52, 124.89, 120.85, 119.78, 84.11, 81.58, 75.65, 73.40, 72.02, 63.24, 63.04, 22.39, 22.26; IR ($CHCl_3$) ν_{max} 3385, 2922, 2851, 2346, 1738, 1729, 1608, 1653, 1485, 1458, 1372, 1329, 1196, 1096, 1024 cm^{-1}. ESI-MS: *m/z* 413.3 $[M+H]^+$, 847.8 $[2M+Na]^+$; HRMS: *m/z* 413.1081 calcd for $C_{18}H_{20}O_{11}+H^+$ (413.1078).

4.3.2. 2-(Acetoxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-3,4,8,10-tetrayl tetraacetate (***4a₂***): Dark yellow amorphous powder; HPLC purity: 97.35% (t_R = 5.97 min); m.p. 206-207 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 7.73 (s, 1H), 5.63 (t, *J* = 8 Hz, 1H), 5.15 (t, 8 Hz, 2H), 4.56 (t, J = 10 Hz, 1H), 4.37 (m, 1H), 4.22 (m, 1H), 4.10 (m, 1H), 3.92 (s, 3H), 2.38 (s, 6H), 2.08 (s, 3H), 2.06 (s, 6H); ^{13}C NMR (125 MHz, acetone-d_6): δ 169.93, 169.44, 169.28, 168.26, 167.58, 161.34, 149.99, 144.09, 141.45, 130.09, 123.50, 119.02, 76.65, 76.30, 72.15, 68.35, 61.97, 61.05, 19.85, 19.83, 19.76; IR ($CHCl_3$) ν_{max} 3422, 2925, 2853, 2346, 2111, 1741, 1651, 1484, 1430, 1370, 1328, 1217, 1098, 1034 cm^{-1}. ESI-MS: *m/z* 561.0 $[M+Na]^+$; HRMS: *m/z* 539.1402 calcd for $C_{24}H_{26}O_{14}+H^+$ (539.1395).

4.3.3. 2-(Acetoxymethyl)-4-hydroxy-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-3,8,10-triyl triacetate (***4a₃***): Pale white gummy solid; HPLC purity: 94.59% (t_R = 6.32 min); m.p. 213-215 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 7.35 (s, 1H), 5.45 (t, *J*= 12 Hz, 1H), 4.98-4.87 (m, 2H), 4.31 (t, *J*= 12 Hz, 1H), 4.20 (dd, *J*= 8 Hz, 12 Hz, 1H), 4.07 (dd, *J* = 4 Hz, 8 Hz, 1H), 3.92-3.89 (m, 1H), 3.77 (s, 3H), 2.20 (s, 3H), 1.93 (s, 3H), 1.91 (s, 3H), 1.89 (s, 3H); ^{13}C NMR (125 MHz, acetone-d_6): δ 171.32, 169.96, 169.31, 167.71, 161.93, 151.00, 145.66, 141.35, 123.49, 119.03, 115.72, 76.87, 76.24, 72.11, 68.51, 62.11, 60.37, 19.88, 19.86, 19.81, 19.78; IR ($CHCl_3$) ν_{max} 3585, 3385, 2922, 2851, 2308, 1742, 1609, 1491, 1463, 1370, 1222, 1095, 1037 cm^{-1}. ESI-MS: *m/z* 495.1 $[M-H]^-$; HRMS: *m/z* 497.1300 calcd for $C_{22}H_{24}O_{13}+H^+$ (497.1290).

4.3.4. 2-((Benzoyloxy)methyl)-3,4-dihydroxy-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl dibenzoate (***4b***): yellow powder; HPLC purity: 100.0% (t_R = 3.68 min); m.p. 180-182 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.22 (d, *J*= 4 Hz, 2H), 7.96 (d, *J*= 8 Hz, 2H), 7.82 (d, *J*= 8 Hz, 1H), 7.70 (t, *J*= 8 Hz, 1H), 7.57-7.28 (m, 10H), 6.03 (t, *J*= 8 Hz, 1H), 5.70 (m, 1H), 5.11-5.01 (m, 2H), 4.71 (m, 1H), 4.15-4.11 (m, 1H), 3.93(s, 3H), 3.46 (d, *J*= 12 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.00, 166.97, 166.36, 163.15, 135.66, 134.91, 134.80, 134.55, 131.87, 131.66, 131.39, 131.23, 131.16, 130.89, 130.37, 130.26, 129.98, 129.82, 129.78, 78.71, 77.96, 74.98, 73.95, 70.03, 63.34, 62.88; IR ($CHCl_3$) ν_{max} 3449, 3065, 2925, 2853, 1738, 1602, 1584, 1486, 1451, 1428, 13670, 1328, 1314, 1274, 1244, 1178, 1109, 1067, 1025, 1003 cm^{-1}. ESI-MS: *m/z* 663.70 $[M+Na]^+$; HRMS: *m/z* 641.4215 calcd for $C_{35}H_{28}O_{12}+H^+$ (641.1654).

4.3.5. (8-((2,6-Difluorobenzoyl)oxy)-3,4,10-trihydroxy-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromen-2-yl)methyl 2,6-difluorobenzoate (***4c***): Pale white powder; HPLC purity: 97.13% (t_R = 2.75 min); m.p. 182-183 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (s, 1H), 7.50-7.46 (m, 1H), 7.37-7.33 (m, 1H), 7.01 (t, *J*= 8 Hz, 2H), 6.89(t, *J*= 8 Hz, 2H), 5.08-5.00 (m, 2H), 4.57-4.53 (m, 1H), 4.24-4.10 (m, 4H), 3.94 (s, 3H), 3.83-2.78 (m, 2H); ^{19}F NMR (376 MHz, $CDCl_3$): δ -108.60 (s, 2F), -109.27 (s, 2F); ^{13}C NMR (125 MHz, acetone-d_6): δ 161.07 (d, $^1J_{CF}$ = 141 Hz), 161.83, 161.78, 161.43, 159.79 (d, *J* = $^1J_{CF}$ =123 Hz), 159.74, 159.45, 159.40, 148.86, 144.87, 143.68, 134.82 (d, $^2J_{CF}$ = 20 Hz, 1C), 134.74, 134.0 (d, $^2J_{CF}$ = 21.4 Hz), 133.91, 122.77, 118.86, 115.96, 112.61, 112.41, 112.21, 79.55, 79.15, 74.34, 73.18, 70.25, 64.47, 59.91; IR ($CHCl_3$) ν_{max} 3396, 3022, 2925, 2852, 2346, 1737, 1626, 1589, 1504, 1471, 1333, 1290, 1252, 1235, 1096, 1057, 1015 cm^{-1}. ESI-MS: *m/z* 609.50 $[M+H]^+$; HRMS: *m/z* 609.0991 calcd for $C_{28}H_{20}F_4O_{11}+H^+$ (609.1015).

4.3.6. 2-(((4-(Tert-butyl)benzoyl)oxy)methyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-3,4,8,10-tetrayl tetrakis(4-(tert-butyl)benzoate) (***4d₁***): White powder; HPLC purity: 99.65% (t_R = 3.52 min); m.p. 182-183 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15 (d, *J* = 8 Hz, 2H), 8.05 (d, *J* = 8 Hz, 2H), 7.91(d, *J* = 8 Hz, 5H), 7.76 (d, *J* = 12 Hz, 2H), 7.56 (d, *J* = 12 Hz, 2H), 7.50 (d, *J* = 8 Hz, 2H), 7.46 (d, *J* = 8 Hz, 2H), 7.38 (d, *J* = 8 Hz, 2H), 7.32 (d, *J* = 8 Hz, 2H), 6.01 (t, *J* =12 Hz, 1H), 5.69 (m, 1H), 5.08 (m, 1H), 4.70 (t, *J* = 8 Hz, 1H), 4.08-3.97 (m, 1H), 3.92 (s, 3H), 3.27 (m, 1H), 1.37-1.26 (m, 45H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.46, 165.49, 165.35, 164.78, 161.78, 158.02, 157.48, 157.03, 156.53, 144.54, 130.33, 130.09, 129.89, 129.72, 129.57, 126.87, 126.51, 12627, 126.05, 125.86, 125.78, 125.56, 125.47, 125.35, 125.32, 125.27, 77.28, 76.71, 73.54, 72.34, 68.24, 61.78, 60.93, 35.27, 35.17, 35.09, 35.04, 35.03, 31.14, 31.07, 31.03, 31.00, 30.94; IR ($CHCl_3$) ν_{max} 2964, 2907, 2870, 2346, 1932, 1738, 1733, 1610, 1573, 1485, 1463, 1428, 1365, 1330, 1314, 1265, 1189, 1111, 1017 cm^{-1}. ESI-MS: *m/z* 1129.40 $[M+H]^-$; HRMS: *m/z* 1129.4041 calcd for $C_{69}H_{76}O_{14}+H^-$ (1129.5308).

4.3.7. 2-(((4-(Tert-butyl)-benzoyl)oxy)methyl)-3,4-dihydroxy-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl-bis(4-(tert-butyl)benzoate) (***4d₂***): White powder; HPLC purity: 99.69% (t_R = 3.58 min); m.p. 182-183 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (d, *J*= 8 Hz, 2H), 8.03-7.99 (m, 2H), 7.94 (d, *J*= 8 Hz, 2H), 7.94-7.92 (m, 1H), 7.56 (d, *J*= 8 Hz, 2H), 7.50 (d, *J*= 8 Hz, 2H), 7.46 (d, *J*= 12 Hz, 2H), 5.59 (t, *J*= 8 Hz, 1H), 4.97-4.95 (m, 1H), 4.57 (t, *J*= 12 Hz, 1H), 4.46-4.40 (m, 1H), 3.97 (s, 3H), 3.85-3.70 (m, 1H), 3.55-3.53 (m, 1H), 3.29-3.14 (m, 1H), 1.38-1.33 (m, 27H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.48, 162.05, 162.00, 158.04, 157.27, 157.23, 157.00, 150.24, 144.43, 141.37, 130.34, 130.19, 130.02, 129.81,

129.71, 129.58, 126.73, 126.68, 126.39, 125.99, 125.78, 125.61, 125.43, 125.39, 123.37, 118.69, 79.02, 75.51, 73.31, 72.93, 68.69, 61.76, 61.39, 60.40, 35.28, 35.27, 35.15, 35.13, 35.11, 31.12, 31.08, 31.00; IR ($CHCl_3$) ν_{max} 3455, 2964, 2870, 1743, 1609, 1573, 1485, 1462, 1427, 1410, 1365, 1330, 1314, 1261, 1188, 1110, 1063, 1016 cm^{-1}. ESI-MS: *m/z* 807.3 $[M-H]^-$; HRMS: *m/z* 809.3499 calcd for $C_{47}H_{52}O_{12}+H^+$ (809.3532).

4.3.8. 9-Methoxy-2-(((3-methylbutanoyl)oxy)methyl)-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-3,4,8,10-tetrayl tetrakis(3-methylbutanoate) (***4e***): Creamish powder; HPLC purity: 98.88% (t_R = 17.85 min); m.p. 182-183 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.70 (s, 1H), 5.56 (t, *J*= 10 Hz, 1H), 5.18 (t, *J*= 10 Hz, 1H), 4.79 (d, *J*= 15 Hz, 1H), 4.32-4.27 (m, 2H), 4.04 (dd, *J*= 5 Hz, 15 Hz, 1H), 3.84 (s, 3H), 3.75 (d, *J*= 5 Hz, 1H), 2.46-2.44 (m, 3H), 2.70-1.98 (m, 12H), 1.04-1.02 (m, 12H), 0.95-0.84 (m, 18H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.56, 171.92, 171.45, 170.44, 169.81, 161.57, 150.11, 144.28, 141.57, 129.55, 123.99, 118.79, 77.33, 77.08, 76.92, 7682, 76.78, 72.89, 71.62, 67.60, 61.41, 61.35, 43.06, 43.00, 42.91, 42.67, 31.43, 30.17, 29.68, 25.75, 25.64, 25.52, 25.36, 22.41, 22.35, 22.33, 22.30; IR ($CHCl_3$) ν_{max} 2960, 2873, 1746, 1609, 1484, 1467, 1426, 1370, 1327, 1293, 1246, 1183, 1165, 1093, 1018 cm^{-1}. ESI-MS: *m/z* 663.3 $[M\text{-isovaleryl}]^-$; HRMS: *m/z* 665.3162 calcd for $C_{39}H_{56}O_{14}$-$C_5H_8O^+$ (665.3168).

4.3.9. 2-((Hexanoyloxy)methyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-3,4,8,10-tetrayl tetrahexanoate (***4f***): Yellow liquid; HPLC purity: 97.99% (t_R = 4.84 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (s, 1H), 5.53 (t, *J*=12 Hz, 1H), 5.20 (t, *J*= 12 Hz, 1H), 4.84 (d, *J*= 12 Hz, 1H), 4.37-4.26 (m, 2H), 4.15-4.11 (m, 1H), 3.88 (s, 3H), 3.81-3.79 (m, 1H), 2.62 (t, *J*= 4 Hz, 4H), 2.37-2.27 (m, 6H), 1.79-1.71 (m, 4H), 1.62-1.58 (m, 6H), 1.40-1.29 (m, 20H), 0.95-0.86 (m, 15H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.27, 172.67, 172.22, 171.15, 170.54, 161.64, 150.02, 144.28, 141.52, 129.50, 123.91, 118.71, 76.77, 76.71, 71.86, 71.78, 67.74, 61.42, 33.96, 33.89, 33.87, 33.85, 33.70, 31.17, 31.14, 31.12, 24.47, 24.38, 24.31, 22.35, 22.26, 13.84, 13.81; IR ($CHCl_3$) ν_{max} 2956, 2933, 2863, 2873, 1748, 1755, 1610, 1577, 1485, 1462, 1426, 1378, 1327, 1294, 1273, 1243, 1218, 1166, 1097, 1017 cm^{-1}. ESI-MS: *m/z* 842.00 $[M+Na]^+$; HRMS: *m/z* 819.4540 calcd for $C_{44}H_{66}O_{14}+H^+$ (819.4525).

4.3.10. 3,4,10-Trihydroxy-9-methoxy-6-oxo-2-(((2,4,6-trinitrobenzoyl)oxy)methyl)-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromen-8-yl 2,4,6-trinitrobenzoate (***4g***): yellowish powder; HPLC purity: 99.98% (t_R = 2.62 min); m.p. 172-173 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.92 (s, 1H), 7.52-7.44 (m, 4H), 5.51 (d, *J*= 12 Hz, 1H), 4.91 (s, 1H), 4.69-4.66 (m, 1H), 4.02-3.94 (m, 5H), 3.71-3.57 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 164.29, 162.04, 161.96, 153.17, 153.09, 148.90, 143.32, 143.10, 123.41, 120.66, 117.78, 117.31, 107.70, 107.65, 75.45, 69.62, 68.19, 65.72, 62.24, 61.10, 60.98; IR ($CHCl_3$) ν_{max} 3384, 3015, 2926, 2851, 2346, 1738, 1653, 1590, 1503, 1486, 1463, 1417, 1334, 1233, 1206, 1170, 1128, 1105, 1014 cm^{-1}. ESI-MS: *m/z* 806.50 $[M]^+$; HRMS: *m/z* 806.1210 calcd for $C_{28}H_{18}N_6O_{23}^+$ (806.0423).

4.3.11. 4-Hydroxy-9-methoxy-3-((2-methylbenzoyl)oxy)-2-(((3-methylbenzoyl)oxy)methyl)-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl bis(3-methylbenzoate) (***4h***): Cream amorphous powder; HPLC purity: 99.90% (t_R = 19.48 min); m.p. 148-150 °C; ^{1}H NMR (500 MHz, acetone-d_6): δ 7.90-7.82 (m, 4H), 7.65-7.60 (m, 4H), 7.51-7.49 (m, 2H), 7.43-7.41 (m, 1H), 7.36-7.32 (m, 1H), 7.27-7.08 (m, 5H), 6.01 (t, *J*= 10 Hz, 1H), 5.67-5.62 (m, 1H), 5.22-5.21 (m, 1H), 4.86-4.82 (m, 1H), 4.29-4.13 (m, 1H), 3.95-3.88 (m, 1H), 3.80 (s, 3H), 3.46-3.34 (m, 1H), 2.29 (s, 3H), 2.21 (s, 3H), 2.16 (s, 3H), 2.12 (s, 3H); ^{13}C NMR (125 MHz, acetone-d_6): δ 165.35, 165.22, 164.88, 164.20, 161.31, 138.31, 138.25, 134.13, 134.11, 133.72, 130.58, 130.54, 130.03, 129.97,128.88, 128.41, 128.29, 127.37, 126.78, 126.69, 77.09, 76.00, 72.85, 69.13, 61.81, 61.44, 20.40, 20.37, 20.30, 20.24; IR ($CHCl_3$) ν_{max} 3385, 2922, 2852, 2346, 1738, 1654, 1608, 1590, 1485, 1458, 1373, 1329, 1270, 1188, 1105, 1080, 1016 cm^{-1}. ESI-MS: *m/z* 801.25 $[M+H]^+$; HRMS: *m/z* 801.2561 calcd for $C_{46}H_{40}O_{13}+H^+$ (801.2542).

4.3.12. (3,4,8,10-Tetrahydroxy-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromen-2-yl)methyl 4-nitrobenzoate (***4i$_1$***): Dark yellow amorphous powder; HPLC purity: 94.71% (t_R = 21.47 min); m.p. 202-205 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 7.80 (s, 1H), 7.80 (s, 2H), 7.59 (s, 2H), 5.72 (t, *J*= 12 Hz, 1H), 5.25 (d, *J* = 12 Hz, 1H), 5.14 (t, *J*= 12 Hz, 1H), 4.70 (t, *J*= 8 Hz, 1H), 4.51-4.50 (m, 1H), 4.39-4.26 (m, 2H), 4.03 (s, 3H); ^{13}C NMR (125 MHz, acetone-d_6): δ 170.30, 164.02, 151.34, 144.57, 138.30, 137.90, 137.07, 133.51, 133.07, 129.62, 129.09, 124.74, 77.82, 72.78, 72.57, 69.91, 64.97, 62.99; IR ($CHCl_3$) ν_{max} 3585, 3473, 3081, 2926, 2854, 1748, 1673, 1609, 1578, 1550, 1484, 1453, 1426, 1371, 1325, 1266, 1240, 1193, 1156, 1102, 1039 cm^{-1}. ESI-MS: *m/z* 495.19 $[M+H]^+$; HRMS: *m/z* 495.1271 calcd for $C_{21}H_{19}NO_{20}+K^+$ (495.1246).

4.3.13. 3,4,10-Trihydroxy-9-methoxy-2-(((4-nitrobenzoyl)oxy)methyl)-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromen-8-yl 4-nitrobenzoate (***4i$_2$***): Creamish amorphous powder; HPLC purity: 91.16% (t_R = 2.76 min); m.p. 191-193 °C; ^{1}H NMR (500 MHz, acetone-d_6): δ 8.92 (t, *J*= 5 Hz, 1H), 8.83 (t, *J*= 5 Hz, 1H), 8.59-8.58 (m, 2H), 8.52-8.49 (m, 2H), 7.97 (t, J= 10 Hz, 1H), 7.89 (t, J= 10 Hz, 1H), 7.49 (s, 1H), 5.34 (d, J= 10 Hz, 1H), 5.16 (dd, *J*= 5 Hz, 20 Hz, 2H), 4.64-4.60 (m, 1H), 4.34-4.26 (m, 2H), 4.04-3.90 (m, 1H), 3.91 (s, 3H); ^{13}C NMR (125 MHz, acetone-d_6): δ 164.22, 162.55, 162.13, 148.68, 148.57, 148.48, 144.89, 144.07, 135.77,135.33, 131.66, 130.70, 130.42, 128.29, 127.72, 124.55, 124.01, 122.71, 118.73, 116.34, 79.51, 79.18, 74.41, 73.19, 70.61, 64.64, 60.04; IR ($CHCl_3$) ν_{max} 3585, 3374, 3092, 2924, 2854, 2346, 1738, 1731, 1653, 1616, 1586, 1533, 1481, 1455, 1351, 1292, 1249, 1114, 1092, 1016 cm^{-1}. ESI-MS: *m/z* 627.45 $[M+H]^+$; HRMS: *m/z* 627.1099 calcd for $C_{28}H_{22}NO_{15}+H^+$ (627.1093).

4.3.14. 9-Methoxy-2-((octanoyloxy)methyl)-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-3,4,8,10-tetrayl tetraoctanoate (***4j***): Yellow liquid; HPLC purity: 98.18% (t_R = 23.19 min); ^{1}H NMR (400 MHz, acetone-d_6): δ 7.23 (s, 1H), 5.66 (t, *J*=12 Hz, 1H), 5.23-5.14 (m, 2H), 4.57 (t, *J*= 12 Hz, 1H), 4.37-4.33 (m, 1H), 4.23-4.19 (m, 1H), 4.12-4.08 (m, 1H), 3.90 (s, 3H), 2.73 (t, J= 8 Hz, 4H), 2.42-2.28 (m, 6H), 1.80-1.72 (m, 4H), 1.67-1.59 (m, 6H), 1.38-1.29 (m, 40H), 0.93-0.87 (m, 15H); ^{13}C NMR (100 MHz, acetone-d_6): δ 173.37, 172.90, 172.60, 171.80, 171.14, 162.12, 150.94, 145.16, 142.62, 130.95, 124.35, 120.00, 77.80, 77.39, 73.20, 72.83, 69.01, 62.55, 61.95, 34.48, 32.45, 32.42, 29.74, 29.71, 29.65, 25.56, 25.49, 25.48, 25.47, 23.28, 23.25, 14.32, 14.30; IR ($CHCl_3$) ν_{max} 3585, 3473, 2956, 2928, 2857, 1748, 1609, 1484, 1462, 1426, 1378, 1326, 1264, 1217,

1194, 1161, 1100, 1018 cm^{-1}. ESI-MS: *m/z* 959.45 $[M+H]^+$; HRMS: *m/z* 976.6373 calcd for $C_{54}H_{86}O_{14}+NH_4^+$ (976.6390).

4.3.15. 3,4,10-Trihydroxy-9-methoxy-6-oxo-2-(((2,4,6-trichlorobenzoyl)oxy)methyl)-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromen-8-yl 2,4,6-trichlorobenzoate (***4k***): Pale yellow amorphous powder; HPLC purity: 99.74% (t_R = 6.36 min); m.p. 165-167 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 8.31 (s, 1H), 7.63 (s, 2H), 7.54 (s, 2H), 5.23 (d, *J*= 12 Hz, 1H), 5.05-4.95 (m, 2H), 4.51 (q, J= 8 Hz, 16 Hz, 1H), 4.19-4.06 (m, 2H), 3.89-3.87 (m, 1H), 3.84 (s, 3H), 3.62-3.57 (m, 1H); ^{13}C NMR (100 MHz, acetone-d_6): δ 165.06, 163.41, 162.80, 150.32, 146.60, 144.57, 138.36, 137.81, 134.01, 133.66, 133.55, 132.57, 129.98, 129.71, 124.40, 120.29, 117.08, 80.96, 80.41, 75.74, 74.60, 71.54, 66.45, 61.47; IR ($CHCl_3$) ν_{max} 3391, 2926, 2346, 2100, 1736, 1653, 1579, 1550, 1507, 1457, 1370, 1332, 1273, 1247, 1190, 1157, 1106, 1037, 1004 cm^{-1}. MS: *m/z* 744.04 $[M+H]^+$; HRMS: *m/z* 759.9294 calcd for $C_{28}H_{18}O_{11}Cl_6+H^+$ (759.9290).

4.3.16. 3,4,10-Trihydroxy-9-methoxy-8-propoxy-2-(propoxymethyl)- 2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (***5a***): Cream powder; HPLC purity: 99.9% (t_R = 2.73 min); m.p. 162-163 °C; ^{1}H NMR (500 MHz, CD_3OD): δ 7.42 (s, 1H), 4.86 (t, *J*= 20 Hz, 1H), 4.05-3.90 (m, 6H), 3.92 (s, 3H), 3.85-3.81 (m, 2H), 3.61-3.57 (m, 1H), 3.52-3.49 (m, 1H), 1.90-1.77 (m, 4H), 1.12-1.05 (m, 6H); ^{13}C NMR (125 MHz, CD_3OD): δ 164.61, 153.10, 150.42, 148.69, 126.29, 119.00, 110.18, 81.07, 80.32, 75.77, 74.51, 74.84, 70.33, 69.95, 60.91, 60.22, 23.09, 22.18, 9.53, 9.45; IR ($CHCl_3$) ν_{max} 3355, 2964, 1937, 2878, 1733, 1711, 1593, 1486, 1473, 1453, 1428, 1387, 1358, 1331, 1178, 1129, 1112, 1030 cm^{-1}. ESI-MS: *m/z* 411.1 $[M-H]^-$; HRMS: *m/z* 413.1807 calcd for $C_{20}H_{28}O_9+H^+$ (413.1806).

4.3.17. 8-Butoxy-2-(butoxymethyl)-3,4,10-trihydroxy-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (***5b***): Pale white needle shaped crystals; HPLC purity: 99.88% (t_R = 3.13 min); m.p. 155-158 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 7.18 (s, 1H), 7.57 (t, *J*= 4 Hz, 1H), 3.86-3.70 (m, 6H), 3.67 (s, 3H), 3.36-3.57 (m, 2H), 3.38 (t, *J*= 12 Hz, 1H), 3.29-3.25 (m, 1H), 1.62-1.49 (m, 4H), 1.36-1.25 (m, 4H), 0.78-0.75 (m, 6H); ^{13}C NMR (125 MHz, CD_3OD): δ 164.59, 153.09, 150.41, 148.67, 126.29, 119.00, 110.14, 81.06, 80.29, 74.50, 73.94, 71.83, 69.90, 68.51, 60.88, 60.21, 32.05, 30.97, 18.99, 18.86, 12.95, 12.79; IR ($CHCl_3$) ν_{max} 3384, 2958, 2935, 2873, 2346, 1715, 1595, 1486, 1454, 1427, 1384, 1361, 1332, 1306, 1242, 1190, 1179, 1130, 1108, 1035 cm^{-1}. ESI-MS: *m/z* 441.5 $[M+H]^+$; HRMS: *m/z* 441.2119 calcd for $C_{22}H_{32}O_9+H^+$ (441.2119).

4.3.18. 3,4,10-Trihydroxy-9-methoxy-8-(pentyloxy)-2-((pentyloxy)methyl)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (***5c***): yellow powder; HPLC purity: 96.48% (t_R = 3.84 min); m.p. 170-172 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 7.28 (s, 1H), 4.72-4.68 (m, 1H), 3.99-3.82 (m , 6H), 3.80 (s, 3H), 3.74-3.70 (m, 2H), 3.51 (t, *J*= 12 Hz, 1H), 3.41-3.37 (m, 1H), 1.77-1.65 (m, 4H), 1.42-1.28 (m, 8H), 0.88 (t, *J*= 8 Hz, 6H); ^{13}C NMR (125 MHz, CD_3OD): δ 166.61, 153.10, 150.42, 148.69, 126.28, 119.02, 110.17, 81.07, 80.32, 74.50, 74.21, 71.85, 69.89, 68.81, 60.87, 60.21, 29.63, 28.56, 28.02, 27.92, 22.24, 22.09, 13.07, 12.99; IR ($CHCl_3$) ν_{max} 3390, 2956, 2933, 2872, 1728, 1594, 1486, 1469, 1454, 1427, 1381, 1361, 1333, 1237, 1193, 1177, 1130, 1108, 1031 cm^{-1}. ESI-MS: *m/z* 469.6 $[M+H]^+$; HRMS: *m/z* 469.2412 calcd for $C_{24}H_{36}O_9+H^+$ (469.2432).

4.3.19. 8-(Benzyloxy)-2-((benzyloxy)methyl)-3,4,10-trihydroxy-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (***5d***): White amorphous powder; HPLC purity: 99.86% (t_R = 2.84 min); m.p. 151-153 °C; ^{1}H NMR (400 MHz, CD_3OD+$CDCl_3$): δ 7.57 (s, 1H), 7.49-7.25 (m, 10H), 5.23 (t, *J*= 12 Hz, 2H), 5.06 (q, *J*=12 Hz, 16 Hz, 2H), 4.57 (d, *J*= 8 Hz, 1H), 3.94 (t, *J*= 12 Hz, 1H), 3.77 (s, 3H), 3.75-3.65 (m, 3H), 3.52 (t, *J* = 12 Hz, 1H), 3.32-3.28 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ 164.39, 152.67, 149.91, 149.06, 137.54, 136.42, 128.26, 128.01, 127.86, 127.76, 127.44, 118.95, 111.55, 80.98, 80.27, 75.67, 74.51, 71.85, 70.77, 69.71, 60.52, 60.36; IR ($CHCl_3$) ν_{max} 3330, 2922, 2851, 2354, 1715, 1654, 1594, 1483, 1447, 1426, 1330, 1246, 1133, 1104, 1030 cm^{-1}. ESI-MS: *m/z* 509.10 $[M+H]^+$; HRMS: *m/z* 509.1816 calcd for $C_{26}H_{24}O_9+H^+$ (509.1819).

4.4. Determination of log P and log D

Log P and log D was determined as per the recently published protocol.[37] For log P and log D experiments, organic phase consisted of n-octanol saturated with water and PBS (pH 7.4), respectively. The aqueous phase consisted of water and PBS (pH 7.4) saturated with n-octanol. Briefly, in 1.5 mL eppendorf tube, 200 µL of stock solution of compound (1000 µg/mL) was added and volume was made up to 1 mL with 300 µL of presaturated n-octanol and 500 µL of presaturated aqueous phase (water / PBS pH 7.4). The eppendorf tubes were shaken overnight at 500 rpm and centrifuged at 16000 RCF (G force) for 20 min so as to separate aqueous and organic layer. The concentration of compound in organic and aqueous phase was determined by HPLC method. The HPLC analysis employed reversed-phase C18 column (NeoSphere, 5 µm, 250 mm × 4.6 mm, Hexon Laboratories Pvt. Ltd., India) using photodiode detector (SPD-M20A, Prominence, Shimadzu). Isocratic mobile phase consisting of acetonitrile and water in various proportions was used for the analysis. The details of HPLC method of all compounds is given in Table S1. Injection volume was 10.0 µL (SIL-20A HT Prominence auto-sampler) (pump, LC-6AD Shimadzu liquid chromatography). The column oven temperature was kept at 37 ºC (column oven, CTO-10ASVP). Each determination was performed in triplicate.

4.5. In-vitro stability studies

4.5.1. Chemical (non-enzymatic) hydrolysis in different buffers at pH 1.2, 4.0, 6.8 and 7.4

To test stability of compound, different buffers were prepared as per the protocol given in Indian Pharmacopoeia 2007.[38, 39] The buffers consisted of hydrochloric acid buffer pH 1.2, phosphate buffer pH 4.0 and phosphate buffered saline (pH 6.8 and pH 7.4).[34, 40-42] A known concentration of compound in DMSO (120 µg/mL) was incubated in buffers of pH 1.2, 4.0, 6.8 and 7.4 at 37 °C for 24 h. From the incubated solutions, aliquot of 50 µL was removed at two time points i.e. at zero time and after 24 h. These aliquots were quenched by adding 200 µL of ice-cold acetonitrile. The resulting solution was centrifuged at 17000 g for 10 min at 4 °C. The supernatant was analyzed, using HPLC conditions as mentioned in Table 3 and Table S2, to determine AUC (area under the curve) of the corresponding prodrug ester

Table 3
HPLC method of bergenin (**1**) and selected bergenin prodrugs

Compound	Details of method	Retention time (min)	
		Bergenin	Compound
1	Isocratic, mobile phase 80:20- ACN : 0.1% formic acid, flow rate 1.0 mL/min, RP-C8 column (Discovery HS, 5 µm, 250 mm × 4.6 mm), Detection wavelength- 280 nm, run time - 10 min	2.857	-
$4a_2$	Isocratic, mobile phase 70:30- ACN : 0.1% formic acid, flow rate 0.8 mL/min, RP-C8 column (Discovery HS, 5 µm, 250 mm × 4.6 mm), Detection wavelength- 254 nm, run time - 10 min	3.502	5.292
$4a_3$	Isocratic, mobile phase 90:10- ACN : water, flow rate 0.8 mL/min, RP-C8 column (Discovery HS, 5 µm, 250 mm × 4.6 mm), Detection wavelength- 254 nm, run time - 10 min	3.474	4.394
$4d_1$		2.857	4.681
$4d_2$			4.645
$4i_2$	Isocratic, mobile phase 80:20- ACN : 0.1% formic acid, flow rate 1.0 mL/min, RP-C8 column		3.547
4k	(Discovery HS, 5 µm, 250 mm × 4.6 mm), Detection wavelength- 240 nm, run time - 10 min		6.618
5d			3.410

and its metabolite (parent compound i.e. bergenin), if any. The experiment was performed in triplicate for each compound.

4.5.2. Stability in biorelevant media, SGF (pH 1.2) and SIF (pH 6.8)

Biorelevant media *viz.* SGF (pH 1.2) and SIF (pH 6.8) were prepared as per the protocol given in USP 2000 [43]. The stability of compound in biorelevant media *viz.* SGF and SIF was determined as per the protocol mentioned in section 4.5.1. The solution of compound was incubated in SGF and SIF at 37 °C and aliquot was removed at 0 and 24 h. The HPLC analysis was performed to find out degradation/ hydrolysis of ester prodrug to bergenin, if any, using HPLC method as described in Table 3. Each analysis was performed in triplicate.

4.5.3. Stability in rat plasma

The blood was collected from the jugular vein of rat. It was centrifuged at 17000 g for 10 min to get plasma. The stock solution of compound was added to preheated plasma at 37 °C to get concentration of 120 µg/mL. Aliquot of 50 µL was withdrawn at 0 and 4 h. Extraction and analysis methods were similar as mentioned in section 4.5.1 and Table 3. Experiments were performed in triplicate. The time dependent stability of **$4a_2$** in plasma was performed wherein the sampling was done at 0, 15, 30, 120, 240 and 480 min.

4.5.4. Stability in esterase from porcine liver (PLE)

A solution of PLE (150 units/mL) was prepared in PBS (pH 7.4).[44] The stock solution of compound was added to preheated PLE solution at 37 °C to get compound concentration of 120 µg/mL. Aliquot of 50 µL was withdrawn at 0 and 4 h. The analysis was performed in triplicates and as per the method given in Table 3. The time dependent stability of **$4a_2$** in esterase from porcine liver was performed wherein aliquot of 50 µL was withdrawn at 0, 15, 30, 120, 240 and 480 min.

Acknowledgments

R.S. is CSIR-GATE/GPAT-JRF and V.K. is UGC-SRF. S.S.B. is Research associate (Reference No. 45/33/2013/NAN-BMS) receiving financial support from ICMR.

Supplementary Material

Supplementary data associated with this article can be found, in the online version.

References

1. Stella VJ, Prodrugs strategies for improving drug like properties, in: Borchardt RT, Kerns EH, Hageman MJ, et al. (Eds.) Optimizing the "Drug Like" Properties of Leads in Drug Discovery, Springer Science +Business Media, New York, 2006, pp. 221.
2. Guarino VR, Modulating Solubility Through Prodrugs for Oral and IV Drug Delivery, in: Rautio J (Ed.) Prodrugs and Targeted Delivery: Towards Better ADME Properties, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2011, pp. 111.
3. Fellenius E, Berglindh T, Sachs G, et al., *Nature*, 1981; 290: 159.
4. Lindberg P, Nordberg P, Alminger T, et al., *J. Med. Chem.*, 1986; 29: 1327.
5. Cederberg C, Andersson T, Skanberg I, *Scand. J. Gastroenterol. Suppl.*, 1989; 166: 33.
6. Howden C, *Clin Pharmacokinet*, 1991; 2: 38.
7. Hemenway JN, Case Study: Omeprazole (Prilosec®), in: Stella VJ, Borchardt RT, Hageman MJ, et al. (Eds.) Prodrugs, Springer, New York, 2007, pp. 1313.
8. Anonymous, Report on Carcinogens, U.S. Department of Health and Human Services2011.
9. Xu F, Zhang M, Wu Q, et al., *J. Mol. Catal. B: Enzym.*, 2014; 105: 49.
10. Chatterji D, Gallelli J, *J. Pharm. Sci.*, 1979; 68: 822.
11. Wu S, Wang L, Huang X, et al., *Bioorg. Med. Chem.*, 2014; 22: 5234.
12. Cordes EH, in: Cordes EH (Ed.) Hallelujah Moments: Tales of Drug Discovery, Oxford University Press, USA, 2014.
13. Férriz JM, Vinsová J, *Curr. Pharm. Des.*, 2010; 16: 2033.
14. Ashida N, Ijichi K, Watanabe Y, et al., *Biochem. Pharmacol.*, 1993; 46: 2201.
15. Jain S, Singh S, Khajuria A, et al., *J. Med. Chem.*, 2014; 57: 7085.
16. Dhalwal K, Shinde V, Biradar Y, et al., *J. Food Comp. Anal.*, 2008; 21: 496.
17. Singh D, Srivastava S, Govindarajan R, et al., *Acta Chromatogr.*, 2007; 19: 246.

18. Ye Y-P, Sun H-X, Pan Y-J, *Acta Cryst. C*, 2004; C60: 397.
19. Wibowo A, Ahmat N, Hamzah A, et al., *Fitoterapia*, 2011; 82: 676.
20. Newell A, Yousef G, Lila M, et al., *J. Ethnopharmacol.*, 2010; 130: 536.
21. Hwa-Kyung L, Hack-Seang K, Hong-Serck C, et al., *J. Ethnopharmacol.*, 2000; 72: 469.
22. El-Hawary S, Mohammed R, Abouzid S, et al., *J. Pharm. Pharmacol.*, 2016; 68: 834.
23. Srivastava N, Srivastava A, Srivastava S, et al., *J. Liq. Chromatogr. Relat. Technol.*, 2015; 38: 1207.
24. Kumar R, Patel D, Prasad S, et al., *Fitoterapia*, 2012; 83: 395.
25. Jahromi MF, Chansouria J, Ray AB, *Phytother. Res.*, 1992; 6: 180.
26. Wang J, Wang B-j, Wei C-m, et al., *Biomed. Chrom.*, 2009; 23: 199.
27. Takahashi H, Kosaka M, Watanabe Y, et al., *Bioorg. Med. Chem.*, 2003; 11: 1781.
28. Prithiviraj B, Singh UP, Manickam M, et al., *Plant Pathol.*, 1997; 46: 224.
29. He S, Li F, Zhou D, et al., *Drug Dev. Ind. Pharm.*, 2012; 38: 1280.
30. Zhou D, Qin X, Zhang Z-R, et al., *Pharmazie*, 2008; 63: 366.
31. Qin X, Zhou D, Zhang Z-R, et al., *Pharmazie*, 2007; 62: 323.
32. Shi Y-B, Shi Y-P, Meng Q-G, *Biomed. Chromatogr.*, 2006; 20: 1065.
33. Borkar N, Li B, Holm R, et al., *Eur. J. Pharm. Biopharm.*, 2015; 89: 216.
34. Kumar V, Bharate S, Vishwakarma R, *Eur. J. Pharm. Sci.*, 2016; 92: 203.
35. Beresford R, Ward A, *Drugs*, 1987; 33: 31.
36. Wang JJ, Sung KC, Huang JF, et al., *J. Pharm. Pharmacol.*, 2007; 59: 917.
37. Bharate S, Kumar V, Vishwakarma R, *Comb. Chem. High Throughput Screen.*, 2016; 19: 461.
38. *Indian Pharmacopoeia*, 2007; 1: 239.
39. Anonymus, *Indian Pharmacopoeia*, 2007; 1: 239.
40. Mattarei A, Azzolini M, Carraro M, et al., *Mol. Pharm.*, 2013; 10: 2781−2792.
41. Zhang Y, Sun J, Gao Y, et al., *Mol. Pharm.*, 2013; 10: 3195−3202.
42. Yan Y-D, Kim H-K, Seo K-H, et al., *Mol. Pharm.*, 2010; 7: 2132.
43. Anonymus, *The United States Pharmacopoeia*, 2000; 2724.
44. Legigan T, Clarhaut J, Renoux B, et al., *Eur. J. Med. Chem.*, 2013; 67: 75.