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A Novel Process for the Preparation of Clopidogrel base and its Benzenesulfonic Acid Salt*

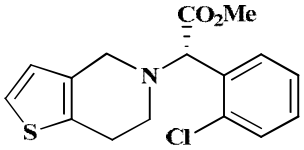
Kaushik Banerjee[1*], Mukul Jain[1], Amar Ballabh[2], Parind Dholakia[1], Mayank Dave[1], Bipin Pandey[3] and Shafiq Sheikh[1]

[1]*Zydus Research Centre, Sarkhej-Bavla N.H 8A Moraiya, Ahmedabad-382210, India.*

[2]*Department of Chemistry, Faculty of Science, M. S. University of Baroda, Vadodara-390002, India.*

[3]*Department of Chemistry, Saurashtra University, University Road, Rajkot-360005, India.*

ABSTRACT: The paper describes a novel crystalline form of the benzenesulfonic acid salt of Clopidogrel (I) and compares some of its pharmaceutical properties with the known solvated forms of the benzenesulfonic acid salt. This new crystalline benzenesulfonate (or besylate) salt is solvent free and more stable than the known forms. A new process for the synthesis of Clopidogrel base **(10)**is also described. The process involves the intra-molecular cyclization of Methyl 2-(2-chlorophenyl)-2-((2-(thiophen-2-yl)ethyl)amino)acetate intermediate of formula **8** using dioxalane to yield Clopidogrel base **(10)**. Resolution of the racemic Clopidogrel base **10** and its subsequent reaction with benzenesulfonic acid in appropriate solvents yielded solvent free crystalline Clopidogrelbenzenesulfonate**(1)**.



Benzenesulfonic acid salt

(1)

KEYWORDS: Clopidogrel; antithrombotic drug; atherosclerosis; o-chlorophenylglycine; 1,3-dioxalane; benzenesulfonic acid salt; Clopidogrelbenzenesulfonate; Clopidogrelbesylate.

Introduction

Methyl α- 5(4,5,6,7-tetrahydro(3,2-c) thienopyridyl) (2-chlorophenyl)-acetate (Clopidogrel) (**10)** is an anti-aggregatory and antithrombotic drug for the treatment and prevention of peripheral vascular, cerebrovascular, and coronary artery diseases[1,2,3]. Clopidogrel when used alone or in combination with Aspirin is known to reduce risks of cardiovascular mortality, nonfatal myocardial infraction and stroke in patients with a history of atherothrombotic diseases[4]. Clopidogrel is an inactive prodrug that is converted to the pharmacologically active metabolite *in vivo* through the hepatic metabolism in order to exhibit the antiplatelet effect[5]. Clopidogrel is first converted by the action of cytochrome P450 (P450) to 2-oxo-clopidogrel (a thiolactone) and then in a second step converted to the pharmacologically active, thiol-containing metabolite[6] (Figure 1). The P450 isoforms involved in the bioactivation of Clopidogrel have been suggested to be CYP1A2 in rats[7]and CYP3A in humans[8], although the contribution of these P450s to produce the active metabolite is still unclear. In addition, several recent clinical studies demonstrated that CYP3A4, CYP3A5, and CYP2C19 have a significant role in the formation of the active metabolite from Clopidogrel[9-13].

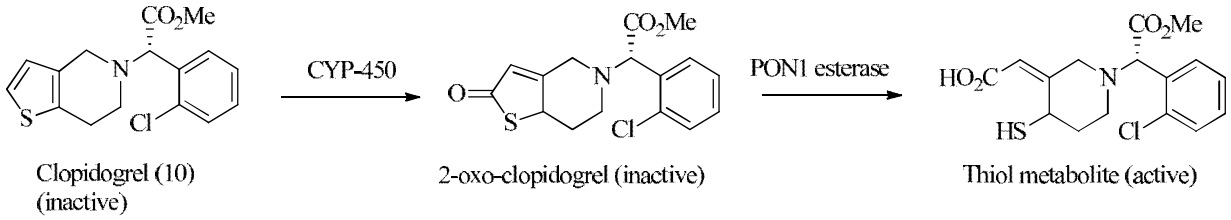
The free-base form of compound (**10**) is unstable owing to a labile proton at the chiral center and is susceptible to racemization[14]. Therefore, the freebase (**10**) must be stabilized through salt formation by reacting with appropriate acids. Clopidoogrelis commercially available as the bisulfate salt. The bisulfate salt was first disclosed in US patent no. 4847265[15]. Several polymorphic forms of the bisulfate salt are already described in the literature. Commercially, it is available in the polymorphic form 2.Several alternate routes for synthesis of Clopidogrel have been reported in the recent literature. Some of them include the use of catalytic asymmetric Strecker reaction[16] or Mannich-like multicomponent synthesis[17]. Biosynthesis of certain key intermediates used in the synthesis of Clopidogrel has also been reported[18-19].

* *For correspondence:* Kaushik Banerjee,

Tel.: +91-2717-665555; Fax: +91-2717-665355;

E-mail: kaushik.banerji@zyduscadila.com

*ZRC communication 464

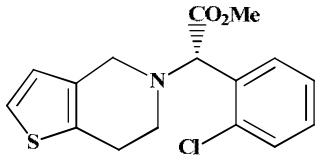


Clopidogrel (10) (inactive) — 2-oxo-clopidogrel (inactive) — Thiol metabolite (active)

Fig. 1 Metabolic pathway of Clopidogrel.

(S)-o-chlorophenylglycine is an important intermediate in the preparation of Clopidogrel **10**. Several processes have been reported for the preparation of this key intermediate and its subsequent conversion to the open chain compound **8**[20-24]. However, many of these processes use chemicals known to be strongly lachrymatory and mucous membrane irritants[25, 26]. Use of such chemicals poses difficulties during handling and up-stream processing; further these chemicals are also unfavorable for human health and environment.

Although, Clopidogrel is a valuable therapy, non-responsiveness or poor responsiveness to Clopidgrel therapy is widely prevalent leading to higher risk of death, myocardial infarction and stroke, a phenomenon referred to as Clopidogrel resistance[27]. Also, a major drawback of the bisulfate salt is that there is an increase in the amount of the inactive metabolite, (+)-(S)-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]-pyridine-5(4H)-acetic acid[28,29] over time. This may further contribute to the variability in effectiveness of different Clopidogrel bisulfate preparations available commercially[30,31]. Therefore, attempts have been made to prepare new salts of Clopidogrel which may provide certain advantages over the bisulfate salt. The patent, US 4847265, describes attempts to prepare several acid addition salts of Clopidogrel **10**. This patent describes the preparation of certain inorganic salts in crystalline form but the carboxylic acid and sulfonic acid salts prepared were either hygroscopic or amorphous in nature. One of the sulfonic acid salt attempted was the benzenesulfonate. Certain solvated forms of the benzenesulfonate salt of clopidogrel **10** have been reported in EP 1480985[32]{the toluene and dioxane(1,4dioxane) solvates}. Other organic salts of clopidogrel have also been reported[33-36]. However, none of these salts are reported to have any advantages over the Clopidogrel bisulfate and do not appear to overcome the problems associated with the bisulfate salt discussed above. The present work attempts to solve some of the reported problems with the bisulfate salt by preparing a new crystalline form of the benzenesulfonate salt of Clopidogrel.



Benzenesulfonic acid salt

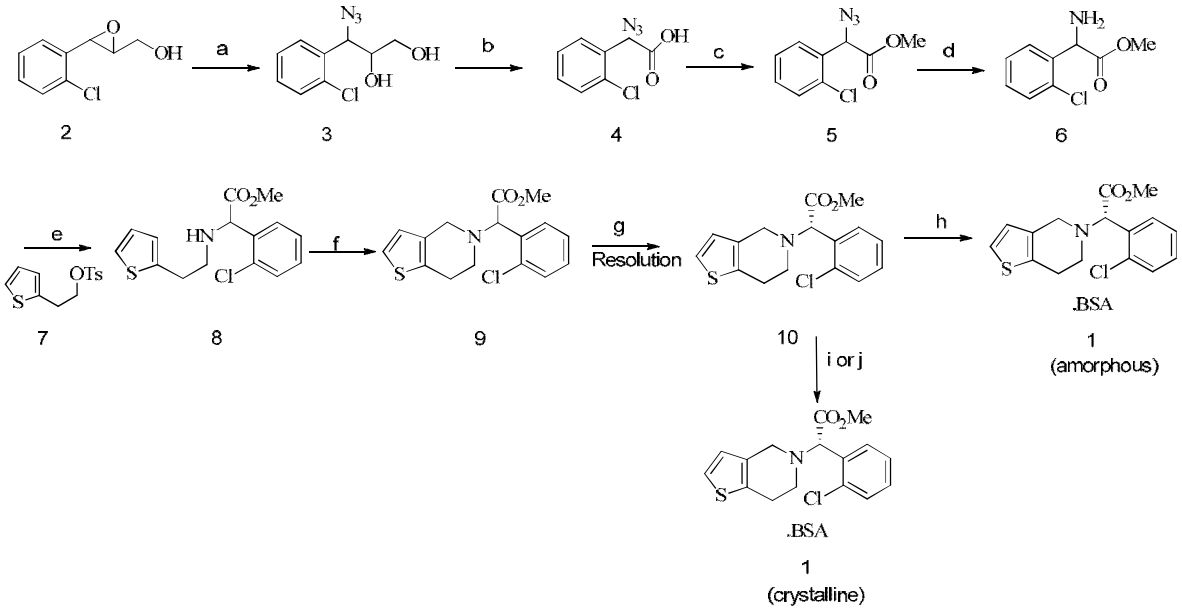
(1)

Fig. 2 Chemical structure of Clopidogrelbenzenesulfonate.

The new crystalline form prepared is a non-solvated form, hereinafter referred to as non-solvated Clopidogrelbenzenesulfonate. The toluene and dioxane solvates were prepared following the processes described in the patent EP 1480985.

Chemistry

Benzenesulfonic acid salt of Clopidogrel was synthesized as described in scheme 1. 2-chloro epoxy cinnamyl alcohol **2** was heated with sodium azide and ammonium bromide in a mixture of ethanol and water to yield the azidodiol derivative **3**[37-39]**.** Oxidation of **3** using sodiummetaperiodate and catalytic amount of $RuCl_3$ in a mixture of acetonitrile, water and carbontetrachloride gave**4**[40,41,42]which was subsequently esterified by treating with thionyl chloride in methanol to give azido ester **5**. Reduction of the azide group under catalytic hydrogenation conditions using palladium on charcoal as catalyst gave the intermediate **6**. Coupling of **6** with **7** in presence of sodium bicarbonate in dimethylformamide gave intermediate **8**. Heating of **8** in 1,3-dioxalane and methanolicHCl at 70 °C resulted in cyclization to give the racemicClopidogrel**9** in quantitative yield. Resolution of **9** using (1S)-(+)-camphor-10-sulfonic acid yielded the Clopidogrel free base**10.** Reacting **10** with benzenesulfonic acid in THF yielded Clopidogrel benzenesulfonate**1** in amorphous form while carrying out the reaction in either isopropanol or, methyl *tert* Butyl ether yielded the salt **1** in crystalline form.



Scheme 1 Reagents and conditions: (a) NaN_3, NH_4Br, ethanol and water, 75 °C, 2 hours (b) sodium metaperiodate, $RuCl_3.H_2O$, acetonitrile, CCl_4, water, room temperature, 6 hours (c) thionyl chloride, MeOH, (d) Pd/C (10%), H_2, MeOH, 54 hours (e) 2-thiophene ethanol tosylate (**7**), $NaHCO_3$, dimethylformamide, reflux, 2 hours (f) methanolicHCl, 1,3-dioxolane, 70 °C, 8 hours (g) (1S)-(+)-camphor-10-sulfonic acid hydrate, acetone, water (h) benzene sulfonic acid, THF. reflux, 10 hours. (i) benzene sulfonic acid, isopropanol 50-55 °C, 20 hours. (j) benzene sulfonic acid, methyl *tert* Butyl ether 50-55 °C, 24 hours.

Table 1 Stability data of solvated and non-solvated forms of BSA salt of clopidogrel at 40 °C ± 2 °C temperature and 75% ± 5% relative humidity.

Sr. No	Type of salt	Test	Time period				
			Initial	3 Days	8 Days	1 Month	2 Month
1	**Toluene solvate of (I).BSA**	Appearance	Off white colored powder	Off white colored powder	Off white colored powder	Light cream colored powder	Light cream colored powder
		Purity by HPLC	99.85 %	99.77 %	99.62 %	98.30 %	92.63 %
2	**Dioxane solvate of (I).BSA**	Appearance	Off white colored powder	Off white colored powder	Light cream colored powder	Light cream colored powder	Light cream colored powder
		Purity by HPLC	99.99 %	99.99 %	99.95 %	99.76 %	99.47 %
3	**Non-solvated form of (I).BSA**	Appearance	Off white colored powder	Off white colored powder	Off white colored powder	Off white colored powder	Off white colored powder
		Purity by HPLC	99.88 %	99.88 %	99.93 %	99.67 %	99.82 %

Results and Discussion

Comparison of stability of the non-solvated Clopidogrel benzene sulfonate, Toluene-Solvate, and Dioxane-Solvate Formulations under accelerated stability study conditions

The stability of the three forms of Clopidogrel benzene sulfonate salt was studied by loading the APIs in a stability chamber at a temperature of 40 °C ± 2 °C and relative humidity of 75% ± 5%. The samples were analyzed after 3 days, 8 days, 1 month and 2 months and the data is provided in table 1. The non-solvated benzene sulfonatesalt remained stable, did not change colour throughout the period of study while, the solvated forms turned to light cream colour. Further, no change in purity was observed for the non-solvated benzene sulfonate salt while there was drop in purity for both the solvated forms.

Dissolution profile and stability data of formulated Clopidogrelbesylate non-solvated form, the toluene solvate and the dioxane solvate.

In another study, the non-solvated Clopidogrel benzene sulfonate, the toluene and dioxane solvates were each formulated into tablets to study their dissolution profile. The results are depicted in Figure 3. The results clearly demonstrated the superior dissolution profile of non-solvated Clopidogrel benzene sulfonate (102.6%) as compared to the solvated forms.

Subsequently these tablets were loaded into a stability chamber at a temperature of 40 °C ± 2 °C and relative humidity of 75%±5%. Tablets containing the solvated forms of Clopidogrelbenzenesulfonate were found to have molted appearance after 3 days which further increased towards the end of study. Also dark brown spots developed on the surface of these tablets. The tablets formulated using the non-solvated Clopidogrelbenzenesulfonate were found to be absolutely normal in appearance throughout the period of study. Subsequently, purity of the samples was estimated by HPLC and the results are depicted as % fall in purity in Figure 4. Thenon-solvated Clopidogrel benzene sulfonate tablets were purer (97.27%) than the solvated forms at the end of the study.

The dissolution profile of the three samples was studied again at the end of 15 days of stability test wherein the dissolution profile of the solvated Clopidogrel tablets was found to have decreased significantly as compared to the non-solvated counterpart on storing for 15 days at a temperature of 40 °C ± 2 °C and relative humidity of 75% ± 5% as shown in Figure 5.

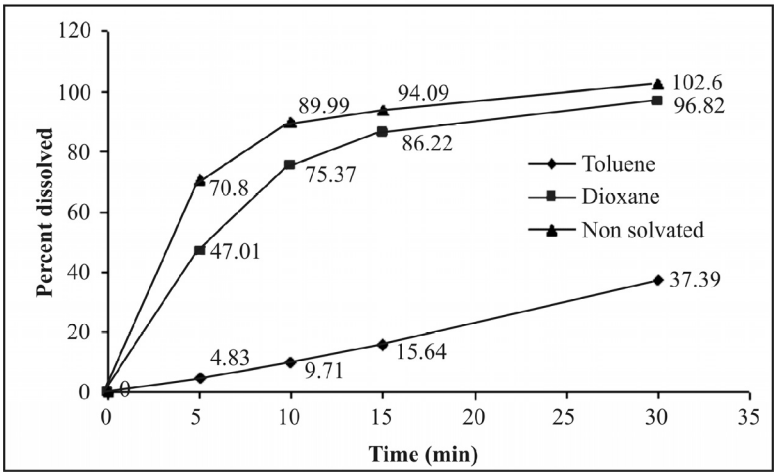


Fig. 3 Dissolution profile of the tablets prepared using the three forms of compound of formula (I) (Initial Analysis).

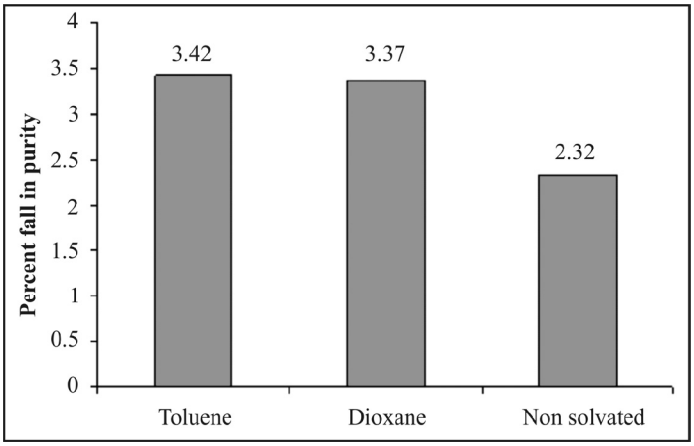


Fig. 4 Percent fall in purity during 15 days stability on 40°C/75 RH.

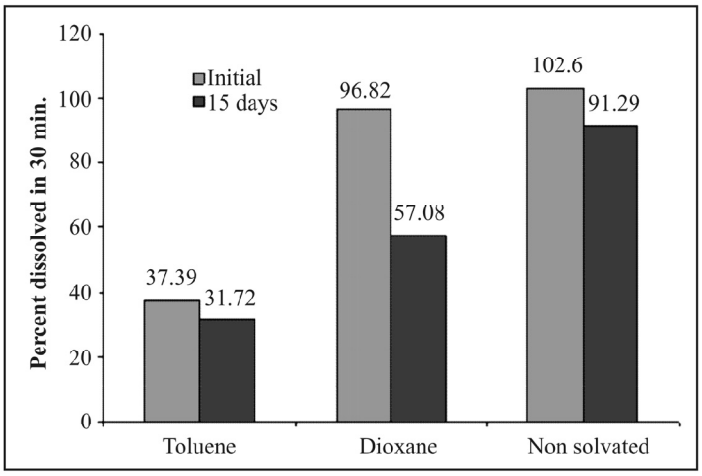


Fig. 5 Dissolution results of the non-solvated form, the Dioxane-solvate and the Toluene solvate forms of Clopidogrelbesylate (1) tablets kept on stability (15 days, 40 °C/75 RH).

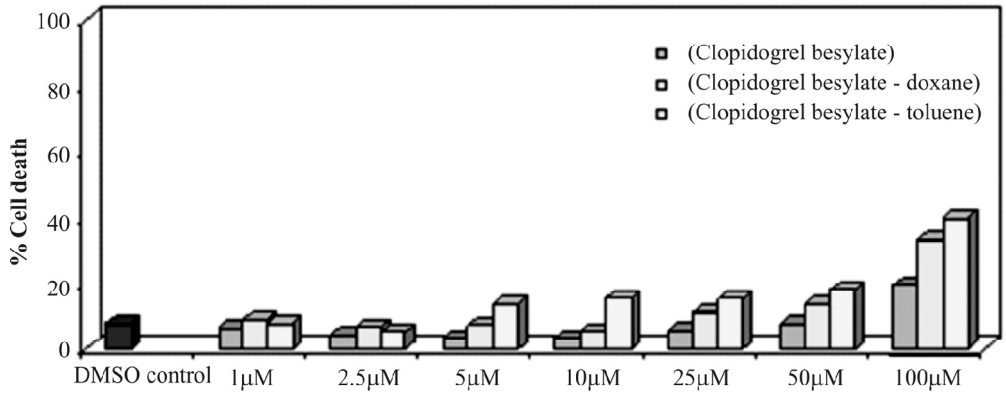


Fig. 6 Cardiotoxicity potential of the non-solvated Clopidogrelbesylate.

Determination of cardiotoxicity potential of the non-solvated, toluene solvate and the dioxane solvate forms of Clopidogrelbenzenesulfonate

In order to compare the safety of the solvated (dioxane and toluence solvates) and the non-solvated forms of Clopidogrel benzene sulfonate, the cardiotoxicity potential of the non-solvated form of Clopidogrel was evaluated in an *in-vitro* set up taking the dioxane-solvate and the toluene solvate forms as positive controls and results are presented in Figure 6. Both the dioxane solvate and the toluene solvate of the benzenesulfonate salt of (I) showed more cardiotoxicity potentialas compared to the non-solvated benzene sulfonatesalt of (I) as evident from the result obtained in *in vitro* study.

The non-solvated form is nontoxic till 50 μM concentrations whereas toluene solvate showed toxicity even at 5 μM concentrations and the dioxane solvate showed toxicity at 25 μM concentrations. Therefore, when either of the dioxane or toluene solvate will be used for chronic therapy, there is a chance that these solvated forms of Clopidogrelbesylate may cause cardiotoxicity.

In another study, the stability of the non-solvated crystalline Clopidogrelbenzenesulfonate described herein, was studied both under accelerated conditions (40 °C/75% R.H.) and long-term. The salt was found to be stable both under stressed condition and upon long term storage. Further, formation of (+)-(S)-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]-pyridine-5(4H)-acetic acid (the inactive metabolite) was not detected in either of the studies. Therefore, it is expected that the variability in effectiveness reported for the Clopidogrel bisulfate will be reduced with this new salt.

Based on the pharmaceutical properties, propensity to cause cardiotoxicity, both the dioxane and toluene solvates of the benzenesulfonic acid salt of formula (I) was not found suitable for further testing in animals.The new non-

solvated form of the benzenesulfonate salt of (I) was found to have good pharmaceutical properties suitable for further development.

In a subsequent study the efficacy of the non-solvated benzenesulfonate salt of formula (I) was compared with bisulfate salt ex-vivo and the results are provided in Table 2.

Table 2 % inhibition of ex-vivo platelet aggregation in rats.

Conc. of ADP (µM)	Time (hours)	% Inhibition of platelet aggregation	
		Non-solvated Clopidogrel besylate of (I)	Clopidogrel bisulfate Form II
5	2	83 ± 5	64± 8
	24	74 ± 8	53±6
	48	60 ± 3	50±6
10	2	70 ± 6	51±7
	24	62 ± 8	35±9
	48	50 ± 5	35±6
20	2	64 ± 6	44±6
	24	52 ± 8	23±8
	48	40 ± 6	20±5
40	2	72 ± 5	45±7
	24	55 ± 7	34±6
	48	52 ± 5	38±5

From the above table it can be concluded that the non-solvated benzenesulfonate salt of formula (I) showed very good anti-platelet efficacy at all time points and ADP concentrations, with maximum efficacy after 2 hours which reduced gradually after 24 & 48 hours.

Conclusion

The crystalline non-solvated benzenesulfonate salt of Clopidogrel(I) exhibits certain beneficial properties and has the potential for mitigating some of the limitations associated with Clopidogrel bisulfate therapy. Studies to further evaluate this non-solvated crystalline Clopidogrelbesylate in patient populations, especially in Clopidogrel resistant patients should be contemplated based on the promising initial data obtained.

Acknowledgement

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Experimental Section

Synthesis

Synthetic materials and methods

Reagents and solvents were obtained from commercial suppliers and used without further purification. Flash chromatography was performed using commercial silica gel (230-400 mesh). Melting points were determined on a capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Shimadzu FT IR 8300 spectrophotometer (Vmax in cm^{-1}, using KBr pellets or Nujol). The ^1H NMR spectra were recorded on a Brucker Avance-300 spectrometer (300 MHz). The chemical shifts (δ) are reported in parts per million (ppm) relative to TMS, either in $CDCl_3$ or DMSO-d_6 solution. Signal multiplicities are represented by s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), bs (broad singlet), and m (multiplet). $^{13}CNMR$ spectra were recorded on Brucker Avance-400 at 100 MHz either in $CDCl_3$ or DMSO-d_6 solution. Mass spectra (ESI-MS) were obtained on Shimadzu LC-MS 2010-A spectrometer. HPLC analysis were carried out at λ_{max} 220 nm using column ODS C-18, 150nm * 4.6 nm * 4 μ on AGILENT 1100 series.

3-(2-chlorophenyl)oxiran-2-yl)methanol(2)

50 gm of cinnamyl alcohol was added to 1.5 litres of dichloromethane taken in a triple neck round bottomed flask at a temperature of 0-5 °C. To the reaction mixture was added 66.54 gm of metachloroperbenzoic acid with stirring, in lots. The temperature was maintained at room temperature. Subsequently, the reaction mixture was stirred at 0 to 5 °C for 3 hours and the stirring was continued slowly at room temperature overnight. The progress of the reaction was monitored by T.L.C., until all the starting material was consumed. To this reaction mixture was added saturated aqueous solution of sodium meta bisulfite, followed by the addition of aqueous NaOH whereby two layers got separated. The organic layer was separated out, while the aqueous layer was re-extracted with 300 ml of dichloromethane. The organic layers were washed with 300 ml. of D. M. water and was dried, concentrated under reduced pressure, and purified by usual procedure yielding the desired epoxy alcohol (7.0 gm).

azido-3-(o-chloro)-propane-1,2-diol(3)

20 gm of epoxy alcohol (**2**) was taken in a round bottomed flask and 35.5 gm of NaN_3 and 6.27 gm of NH_4Br was added to it. To the mixture was added 350 ml ethanol and 60 ml water and it was stirred for 2 hours at 75 °C under reflux. The solvent was evaporated when the salt precipitated out of the reaction mixture. The salt was dissolved in 70 ml of dichloromethane, filtered and the residue was rejected. To the filtrate was added 100 ml of water when two layers got separated. The lower MDC layer

was separated and dried. The solvent was evaporated at 40 °C under reduced pressure to give 20.2 gm of the diol. IR (cm^{-1}) : ($CHCl_3$) 3384 (-OH stretch); 2106 (N_3); ^{1}HNMR ($CDCl_3$): δ 1.96 (t, 1H), 2.4 (d, 1H); 3.7 (t, 2H), 3.9-4.0 (q, 1H); 5.2 (d, 1H), 7.2-7.5 (m, 4H); ^{13}CNMR ($CDCl_3$): δ ppm 62.56, 63.73, 73.06, 127.4, 128.7, 129.7, 130.02, 133.7, 133.91; ESI-MS m/z: 288.1 $(M^+H)^+$, 245.2 $(M^+NH_4)^+$

azido-2-(2-chlorophenyl)acetic acid(4)

In a 1 litre round bottomed flask was taken the 11 gm of azidodiol obtained above and to it was added 92 mL of acetonitrile, 87 mL of CCl_4 and 131 mL D. M. water, when two layers were formed. To the mixture was added 4.0 eq. sodium metaperiodate and 236 mg of $RuCl_3.H_2O$, when the colour changed to brick red. The reaction mixture was stirred for 6 hours at room temperature and kept overnight. To the mixture was added 200 mL of ether, the organic layer formed was filtered out through hyflow bed, washed with water and dried over anhydrous Na_2SO_4 to give 6.0 gm (64.5%) of azido acid. IR (cm^{-1}): ($CHC1_3$) 2928 (NH), 2110 (N_3), 1725 (CO); ^{1}H NMR ($CDC1_3$): 5.59 (s,1H), 7.3-7.36 (m, 2H), 7.42-7.48 (m, 2H), 9.5 (b, 1H); ^{13}C NMR ($CDC1_3$) : δ ppm 61.99, 127.5, 128.87, 130.19, 130.7, 131.5, 134.02, 173.8; ESI-MS m/z: 210 (M^+H^-).

Methyl 2-azido-2-(2-chlorophenyl)acetate(5)

1.0 gm of azido acid obtained above was taken in a round bottomed flask and 5 mL of methanol was added to it and the solution was stirred for 30 minutes. To the reaction mixture was added 0.9 grams of thionyl chloride dropwise. The reaction mixture was stirred for 3 hours at room temperature. The excess solvent was evaporated under reduced pressure. To the residue was added 25 mL MDC, the organic layer formed was washed with a 1% solution of $NaHCO_3$ and then with D. M. water and the organic layer was dried with anhydrous Na_2SO_4. The excess solvent was evaporated under reduced pressure when the azido ester was obtained in ~ 90% yield (0.9 gm). IR (cm^{-1}): 2108 (N_3), 1751.2 (CO); ^{1}H NMR ($CDC1_3$): 3.79 (s, 3H), 5.5 (s, 1H), 7.31-7.46 (m, 4H); ^{13}C NMR ($CDC1_3$): δ ppm 53.03, 62.02, 62.02, 127.3, 128.7, 130.0, 130.4, 132.05, 133.09, 169.06; ESI-MS m/z: 243.2 $(M^+NH_4)^+$.

Methyl 2-amino-2-(2-chlorophenyl)acetate(6)

1 gm of azido ester prepared above was dissolved in 20 mL MeOH and added to the metallic container of Parr apparatus. To it was added Palladium charcoal (10%) and the container was shaken for 54 hours under hydrogen pressure. After completion of the reaction, the reaction mixture was filtered, the solvent evaporated under reduced pressure and 25 mL of MDC added to the reaction mixture. The amino ester obtained was purified by conventional techniques, when 250 gm (28.4%) of the purified product was obtained. IR (Cm^{-1}): 3381 (NH-stretch), 1738 (CO), 1126 (CN stretch); ^{1}H NMR ($CDC1_3$) :δ 2.0 (bs 2H), 3.72 δ (s, 3H, OCH_3), 5.0δ (s, 1H), 7.24-7.28 (m, 2H), 7.33-7.40 (m, 2H) ESI-MS m/z: 200 $(M^+H)^+$.

Methyl 2-(2-chlorophenyl)-2-((2-(thiophen-2-yl)ethyl) amino) acetate(8)

4 gm of the amino ester 6 obtained previously was taken in a round bottomed flask and to it was added 1.4 eq. of 2-thiophene ethanol tosylate 7, 20 mL of dimethyl formamide and 2 eq. of $NaHCO_3$. The mixture was refluxed for 2 hours, kept overnight and the excess solvent was evaporated under reduced pressure. The crude product was purified using conventional techniques to obtain 0.9 gm of 8. IR (cm^{-1}): 3018.4 (NH stretch), 1736 (CO); ^{1}H NMR ($CDC1_3$): δ 2.1 (b, 1H), 3.0 (m, 1H), 3.3 (m, 1H), δ 3.5-3.6 (m, 2H), 5.65 (s, 1H), 6.8 (d, 1H), 6.9 (dd, 1H), 7.1 (dd, 1H), 7.3-7.4δ (m, 3H), 8.0 (dd, 1H); ^{13}C NMR: δ ppm 30.44, 48.89, 52.33; 61.5, 123.5, 124.9, 126.7, 127.2, 128.5, 129.1, 129.7, 134.0, 136.0 and 142, 172.59; ESI-MS m/z: 310.2 $(M^+H)^+$.

Methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c] pyridin-5(4H)-yl)acetate (9)

To 2.0 gm of the HCl salt of the ester 8 obtained above was added 5 mL of 1,3-dioxolane and 0.5 mL of methanolic HCl. The mixture was stirred for 8 hours at 70 °C, the excess dioxolane was evaporated under reduced pressure. The residue was dissolved in 60 mL dichloro-methane, basified with 10% $NaHCO_3$, the organic layer was washed with water and the excess solvent was evaporated under reduced pressure to obtain 1.68 gm (~ 95%) of compound of formula (9) as free base. IR (Cm^{-1}): 1740 (CO); ^{1}H NMR ($CDC1_3$): δ 2.88 (s, 4H), 3.6-3.8 (s, 3H, m, 2H), 4.9 (s, 1H), 6.6 (d, 1H), 7.0 (d, 1H), 7.2-7.7 (m, 4H); ^{13}C NMR ($CDC1_3$) : 25.54, 48.3, 50.6, 52.14, 67.8, 122.7, 125.2, 127.1, 129.4, 129.7, 129.9, 133.2 133.8, 134.7 and 171.34; ESI-MS m/z: 322.1 $(M^+H)^+$.

(S)-(+)-Methyl (2-chlorophenyl)-(6,7-dihydro-4H-thieno[3,2-c]pyrid-5-yl)acetate (10)

2 g (0.0173 mole) Methyl (2-chlorophenyl)-(6,7-dihydro-4H-thieno[3,2-c]pyrid-5-yl)acetate was dissolved in 10 mL acetone and the reaction mixture was stirred for 10 min, followed by reflux. To the reaction mixture, 1.49 g (1S)-(+)-camphor-10-sulfonic acid hydrate in 0.8 mL water was added followed by 1 mL acetone. The whole reaction mixture was then refluxed for 1 hr. and cooled gradually. The mixturewas later stirred overnight at room temperature. The clear solution was cooled further at 0 to -5 °C, wherein precipitate was obtained. The salt formed was added to ethyl acetate and water, which was later basified with $NaHCO_3$, the organic layer was washed with water, concentrated under reduced pressure, to give free base 0.386 g with chiral purity = 99.85 % (+)-isomer (ee = 99.7 %).

IR (Cm^{-1}): 1740 (CO); ^1H NMR ($CDC1_3$): δ 2.88 (s, 4H), 3.6-3.8 (s, 3H, m, 2H), 4.9 (s, 1H), 6.6 (d, 1H), 7.0 (d, 1H), 7.2-7.7 (m, 4H); ^{13} C NMR ($CDC1_3$): 25.54, 48.3, 50.6, 52.14, 67.8, 122.7, 125.2, 127.1, 129.4, 129.7, 129.9, 133.2 133.8, 134.7 and 171.34; ESI-MS m/z: 322.1 $(M^+H)^+$.

Benzenesulfonate salt of (S)-(+) (+)-Methyl (2-chloro-phenyl)-(6,7-dihydro-4H-thieno[3,2-c]pyrid-5-yl)acetate (1) in amorphous form

The compound of formula (I) was dissolved in THF. To it, benzene sulfonic acid was added at 20 ^oC, and the reaction mixture was heated to reflux temperature for 10 hr. The solvent was evaporated to dryness under reduced pressure to obtain the besylate, salt of formula (I) in the amorphous form.

M.P.: 92 ± 3 ºC; XRD: Amorphous; DSC: No melting peak; % water: 0.5-4% by weight (different batches)

Benzenesulfonate salt of (S)-(+) (+)-Methyl (2-chloro-phenyl)-(6,7-dihydro-4H-thieno[3,2-c]pyrid-5-yl)acetate (1) in crystalline form

The base of formula (I) (60 g) was dissolved in isopropanol at 50-55 ^oC. To it was added benzene sulfonic acid (30 g) dissolved in isopropanol at 50-55 ^oC. The reaction mixture was stirred for 20 hr. The solid was filtered and washed with isopropanol and dried in a vacuum oven for at least 20 hr to give the benzenesulfonate salt of formula (I), which on characterization was found to be in crystalline form.

IR (KBr cm-1) 3446, 1749, 1232, 1163, 1016, 765, 727, 613;^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 7.71-7.68 (m, 2H), 7.66-7.51 (m, 4H), 7.45 (d, J = 5.2 Hz, 1H), 7.34-7.27 (m, 3H), 6.88 (d, J = 4.8 Hz, 1H), 5.66 (s, 1H), 4.20 (bs, 2H), 3.76 (s, 3H), 3.4 (bs, 2H), 3.09 (s, 2H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 167.4, 148.2, 134.3, 132.2, 131.7, 130.6, 130.2, 128.5, 127.6, 125.4, 125.1, 65.6, 53.7, 50.3, 40.1, 39.9, 39.7, 39.5, 39.3, 39.1, 38.8, 22.4; ESI-MS m/z (Relative intensities) (+ve mode) 321.9 (M^+H)+ (100%); M.P. 130-135 ^oC; XRD: Crystalline; DSC: 127.5-132.9 ^oC; % water: 0.1-0.3 %.

Benzenesulfonate salt of (S)-(+) (+)-Methyl (2-chloro-phenyl)-(6,7-dihydro-4H-thieno[3,2-c]pyrid-5-yl)acetate (1) in crystalline form

The base of formula (I) (5 g) was dissolved in methyl tertiary butyl ether. To this, benzene sulfonic acid (2.5 g dissolved in methyl tertiary butyl ether was added at 50-55 ^oC. The reaction mixture was seeded with crystalline benzenesulfonate salt of formula (I) (50 mg and the reaction mixture was stirred for at least 24 hr. The solid was filtered and washed with methyl tertiary butyl ether and dried in vacuum oven for at least 20 hr to give benzenesulfonate salt of the compound of formula (I), which on characterization was found to be in crystalline form.

M.P.135 ± 2 ^oC

DSC, TGA and single crystal analysis conclusively proved that the Clopidogrelbenzenesulfonate prepared according to the present process is in non-solvated form.

Dissolution Profile

The benzenesulfonate salt of formula (I) prepared above (non-solvated) as well as the toluene-solvate, and dioxane-solvate were prepared into tablets. The tablets were evaluated for their appearance, purity, dissolution profile and stability. Dissolution was performed using USP Type II dissolution apparatus at 50 rpm, in 900 mL of 0.1N HCl as dissolution media. The samples were collected for analysis at 5, 10, 15 and 30 minutes time points.

At each time point, 5.0 mL of sample was withdrawn and replaced with equal quantity of same buffer solution and the collected samples were analyzed by HPLC method.

In-Vitro Cardiotoxicity Assay

H9C2 cell lines were obtained from NCCS (Pune, India). DMEM media, MTT were obtained from Sigma.H9C2 cells were trypsinized and washed twice with complete DMEM. $2X10^4$ cells were seeded per well in a 96 well plate in a total volume of 200 µL per well. Plates were then kept overnight at 37^oC, 5% CO_2 with humidified atmosphere. Next morning media were removed from each well and cells were washed with 1X PBS and treated with various concentrations of doxorubicin or Clopidogrel benzene sulfonate API(s). Plates were then incubated for 48 hrs at 37^oC, 5% CO_2 with humidified atmosphere. At the end of the incubation period content of all the wells was aspirated and cells were washed with 200 µL of 1X PBS and 100 µL of fully constituted DMEM was added to each well. MTT was added to each well (final concentration 0.5 mg/mL), in dark. The plate was then incubated for 4 hr at 37 ^oC, 5% CO_2 in humidified atmosphere. At the end of the incubation, media containing MTT from each well was removed and 200 µL of Dimethyl sulfoxide (DMSO) was added to each wells. The plates were kept on a plate shaker for 30 minutes at room temperature. The absorbance was measured at 570 nm using the spectrophotometer. Lesser color intensity represents more cell death.

Inhibition of *Ex-vivo* Platelet Aggregation in Rats[43]

All animals were used from inbred colonies which are maintained on standard laboratory rodent chow ad libitum and the study protocols were approved by institutional animal ethics committee. Male Wister Rats of 6-8 weeks age and 200-250 g of body weight were selected for the study. The salt forms were formulated in saline containing 5% ethanol and each rat received 5 mL/kg p.o. volume. The formulations of the salts were always prepared freshly in vehicle. The vehicle (10 mg/kg/5 mL) were administered

to non-fasted rats. At 2, 24 and 48 hr after initial dosing, 8 mL of blood was collected by cardiac puncture (after anaesthetizing the rats) from which the platelet rich plasma was isolated and the platelet aggregation was measured using 2.5 % (w/v) sodium citrate as the coagulant. The main pharmacodynamics measure was the inhibition/ reduction of ADP-induced platelet aggregation of platelet-rich plasma (PRP) which was measured using Whole Blood Aggregometer and the statistical analysis of % aggregation was performed by one-way ANOVA (Analysis of Variance) followed by Bonferroni post hoc test. All the analysis were done using Graph pad Prism software. Results are expressed as mean ± SEM and differences of $P < 0.05$ were considered statistically significant.

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Synthesis and Biological Studies of a Novel CB1 Antagonist *

Authors
K. Banerjee[1,2], M. Jain[1], A. Vallabh[2], B. Srivastava[1], A. Joharapurkar[1], H. Patel[1]

Affiliations
[1] Zydus Research Centre, Ahmedabad, India
[2] Department of Chemistry, Faculty of Science, M. S. University of Baroda, Vadodara, India

Key words
- cannabinoid CB1 and CB2 receptors
- neutral antagonists
- anti-obesity drugs
- piperidin-1-ylamide derivatives

Abstract

This paper describes the synthesis, early process development, salt selection strategies and pre clinical evaluation of novel, potent and selective CB1 antagonist, 8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid piperidin-1-ylamide **1**. The CB1 antagonism of compound **1** is also confirmed by reversal of CB1 agonist-induced hypothermia in Swiss albino mice. The process for the preparation of the compound **1** as a crystalline solid is also described. The crystalline form of the compound is found to be low bioavailable, therefore attempts have been made to improve its bioavailability through polymorphic transformation and salt formation. None of the salts prepared were found to be suitable for further development. The amorphous form of the compound **1** is found to be better suited. In vivo efficacy study of the amorphous form of compound **1** in 5% sucrose solution intake model in female Zucker fa/fa rats at single oral dose of 10 mg/kg demonstrates better reduction in the sucrose solution consumption than the corresponding crystalline form. The plasma concentration C_{max} at AUC exposure of the amorphous form of the compound **1** is significantly improved and better than the C_{max} of the corresponding crystalline form of the compound **1**. On the basis of the efficacy, pharmacokinetic and toxicological evaluations, the compound **1** in the amorphous form is selected as a pre-clinical lead candidate.

Introduction

Obesity is widely recognised as the largest and fastest growing public health problem in the developed and developing countries [1] and considered as a global epidemic. The International Obesity Task Force estimates that more than 300 million individuals world wide are obese and an additional 800 million are over-weight [2,3]. Sedentary life style, advent of junk foods, modern lifestyles with their various stresses and increasing disposable income play a part in this problem [4]. Obesity is associated with substantial increases in morbidity, premature mortality, impaired quality of life and large healthcare costs [5]. The problem with obesity are compounded due to the major comorbidities associated with it that include type 2 diabetes, metabolic syndrome, hypertension, dyslipidaemia, myocardial infarction, stroke, certain types of cancers, sleep apnea and osteoarthritis [6]. Lifestyle changes in the form of dieting and/or exercise per se do not generally produce marked or sustainable weight loss [7,8], whereas psychological therapies are difficult to deliver on a mass scale [9] and long-term results are disappointing. Hence, pharmacotherapy for obesity has become a popular choice, especially among the younger generations [10].

The role of cannabinoid receptor system in the regulation of appetite and food intake in animals and humans intrigued the scientific community to develop drugs targeted towards cannabinoid receptor type 1 (CB1) [11–13]. Rimonabant hydrochloride (Compound-**2**, **Fig. 1**) has been the first therapeutically relevant, potent and selective CB1 receptor inverse agonist, which was approved in Europe as an anti-obesity drug [14]. However, reports of serious psychiatric problems (such as anxiety, depression and suicide) led to withdrawal of Rimonabant and termination of several other CB1 receptor-antagonist-based anti-obesity drug development programmes (such as taranabant, otenabant, surinabant and ibipinabant) [15,16].

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Correspondence
K. Banerjee
Zydus Research Centre
Sarkhej-Bavla N.H 8A Moraiya
Ahmedabad-382210
India
Tel.: +91/2717/665 555
Fax: +91/2717/665 355
kaushik.banerji@zyduscadila.com

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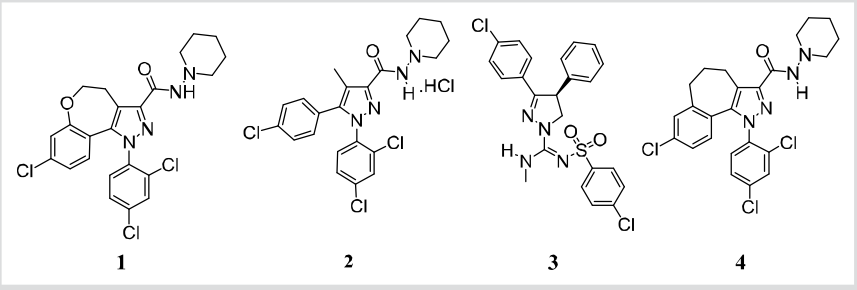

Fig. 1 CB1 receptor antagonist with diverse chemical structures.

Researchers have tried to develop several classes of CB1 receptor antagonist with diverse chemical structures [17–19]. Thus, for example, Solvay Pharmaceuticals has disclosed the 3,4-diaryl dihydropyrazole class of compounds (Compound-**3**, ◻ **Fig. 1**) as a CB1 antagonist, which has elicited potent in vitro [20] and in vivo activities [21]. 8-Chloro-1-(2,4-dichlorophenyl)-*N*-piperidin-1-yl-1,4,5,6-tetrahydrobenzo-[6, 7]-cyclohepta-[1, 2–c]-pyrazole-3-carboxamide (Compound-**4**, ◻ **Fig. 1**) was found to be a very potent CB1 antagonist in cell-based in vitro assays, and ex vivo screens [22]. However, the compound **4** had poor in vivo efficacy and oral bioavailability [23]. Despite the withdrawal of Rimonabant and the demise of several CB1 receptor antagonist development programmes, researchers believe that one has not yet reached the end of the line for anti-obesity treatments targeting the CB1 receptor [24,25]. It is now believed that neutral antagonists might retain the weight loss advantages and will be devoid of adverse effects [26]. Another promising approach is to develop agents which restricts their function through the periphery and do not reach to brain [27]. In continuation of our drug-discovery efforts to develop novel therapeutic agents to treat obesity, we have discovered a series of novel compounds as CB1 modulators [28] and compound **1** (◻ **Fig. 1**) has been selected as a lead compound for pre-clinical evaluation. Though the compound **1** showed good pharmacological profile, it had poor bioavailability. The aim of the present study is to develop a new synthetic process for the preparation of compound **1** in high yield suitable for pre-clinical development and improve its bioavailability through salt selection strategies as well as through polymorphic transformations. Herein, the efficacy, pharmacokinetic evaluations of novel CB1 antagonist, 8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e] azulene-3-carboxylic acid piperidin-1-ylamide **1** are described.

Chemistry

The compound **1** is synthesized as described in ◻ **Fig. 2**. 4-(3-chlorophenyl)-butanoic acid **5** is reacted with oxalyl chloride in dichloromethane at room temperature (27–30 °C) followed by Friedel-craft's acylation to provide 8-Chloro-3,4-dihydro-2H-benzo[b]oxepin-5-one **6**. Treatment of **6** with diethyl oxalate in ethanol followed by 2N HCl afforded 8-Chloro-5-oxo-2,3,4,5-tetrahydro-benzo[b]oxepin-4-yl)-oxo-acetic acid ethyl ester **7**. The reaction of **7** with 2,4-Dichlorophenyl hydrazine hydrochloride in ethanol at 5–7 °C provided 8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid ethyl ester **8**, which upon hydrolysis using KOH in methanol yielded 8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e] azulene-3-carboxylic acid **9**. The reaction of **9** with $SOCl_2$/toluene followed by reaction with 1-Amino piperidine under usual amide bond formation chemistry yielded 8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e] azulene-3-carboxylic acid piperidin-1-ylamide as an oil which was treated with ethereal HCl to get the HCl salt **10** as a brown solid . Trituration with ethyl acetate and subsequent drying yielded the pure compound **10** as a white solid. The neutralization of **10** with NaOH provided the compound **1** in crystalline form. The crystalline compound **1** is dissolved in dimethyl formamide by heating at 40 °C and the resulting solution is cooled to 27–30 °C over a period of 48 h to give needle shaped crystals of the DMF solvate **11.** The different salts of **1** were prepared by dissolving **1** in acetone and adding appropriate mineral/organic acids at 40–50 °C [**12** (a–e)]. Stirring the compound **1** in dichloromethane at room temperature (27–30 °C), followed by removal of solvents under reduced pressure, afforded the amorphous form of the compound **1**.

Results and Discussions

The compound **1** in its crystalline form is synthesized according to the scheme described in ◻ **Fig. 2**. The crystalline nature of the compound **1** was confirmed by XRD (◻ **Fig. 3b**). The binding affinity of compound **1** was tested in an in vitro cAMP assay. The compound **1** exhibits an EC_{50} 14.5μM in human CB1 receptor forskolin-induced cAMP assay. In the human CB2 receptor (cannabinoid receptor 2) binding assay, the compound **1** showed Ki 3.19μM, IC_{50} 4.75μM at 30μM concentration, indicating favorable CB1 selectivity. In vitro CB1 antagonism was measured using a binding assay in CHO cells expressing human CB1 receptors (hCB1). Interestingly, the compound **1** did not change the forskolin-stimulated cAMP accumulation in CB1-transfected HEK cells up to 10μM concentration indicating the possible neutral antagonist nature. The CB1 antagonism of compound **1** was also confirmed by reversal of CB1 agonist-induced hypothermia in Swiss albino mice [29].

However, the crystalline form of the compound **1** like other promising molecules in the CB1 class suffers from poor oral bioavailability and pharmacokinetic profile. In order to improve the oral bioavailability of compound **1**, attempts were made to change the polymorphic form of the compound **1**. However, these attempts were limited due to poor solubility of compound **1** in most of the organic solvents. In one of the attempts, the compound **1** was dissolved in large excess of dichloromethane, heated to reflux and the volume of the resulting solution was reduced to minimum, which upon cooling to 27–30 °C over a period of 48 h did not yield any solid. In another attempt, the compound **1** was dissolved in large excess of acetone, heated to reflux and the volume of the solution reduced to minimum. The residue obtained was cooled to 27–30 °C and kept over a period of 48 h, but did not yield any solid. The compound **1** was found to

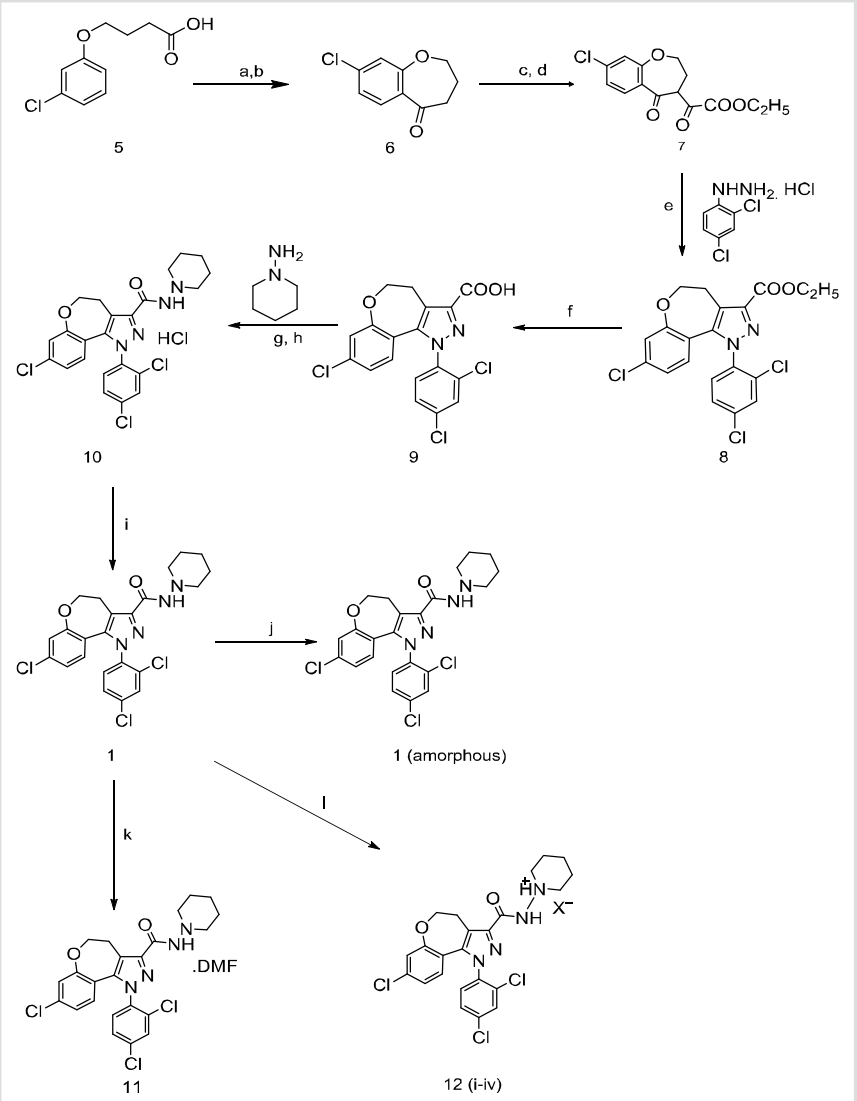

Fig. 2 Synthetic scheme for preparing compound **1**. Reagents and conditions: **a** $(COCl)_2$, anhydrous CH_2Cl_2, stirred at 0 °C for 30 min and 26–28 °C for 1.5 h; **b** anhydrous $AlCl_3$, CH_2Cl_2, stirred at 0–5 °C for 20–25 min and at 26–27 °C for 30 min; **c** diethyl oxalate, Na metal, ethanol; **d** 2N HCl, $CHCl_3$; **e** ethanol, IPA/HCl, refluxed at 75–77 °C for 2 h; **f** KOH/MeOH; **g** $SOCl_2$, toluene, refluxed at 107–108 °C for 30 min; **h** anhydrous methanol, ethereal HCl, 0–5 °C; **i** NaOH; **j** CH_2Cl_2, reflux under reduced pressure at 50 °C; **k** DMF, heat at 40–50 °C for 2 h, cooled and kept for 48 h; **l** acetone, heat at 40 °C till clear solution, added acids corresponding to X=Cl−, HSO4−, $(COO)_2^{-2}$ & $-CH_3I$ in acetone, and the mixture was cooled when necessary and filtered.

be more soluble in solvents such as dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO). The hot solution of compound **1** in DMSO upon cooling at 27–30 °C over a period of 48 h afforded a pasty material, which was found to be the solvate of DMSO. In a subsequent attempt, the solution of compound **1** was prepared in DMF by heating and subsequent cooling at 27–30 °C over a period of 48 h yielded crystals. The crystals of compound **1** in DMF were characterized as solvates of DMF **11**. The acute exposure to dimethyl formamide is reported to cause liver toxicity in humans [30], thus the crystalline form of the DMF solvate of compound **1** was not evaluated further in any pharmacological studies.

Furthermore, we synthesized the stable oxalate **12b**, benzenesulfonate **12c**, bisulfate **12d**, and methyl iodide **12e** salts of compound **1**. None of the salts altered the in vitro binding mode, potency and selectivity as initially exhibited by the crystalline form of compound **1**. The hydrochloride salt **12a** upon storage gets hydrolysed wherein the piperidine ring gets cleaved to form the corresponding methyl ester **13,** ◘ **Fig. 4**. The structure of compound **13** was confirmed through single crystal analysis.

The oxalate **12b**, benzenesulfonate **12c**, bisulfate **12d**, and methyl iodide **12e** salts of compound **1** significantly improved the pharmacokinetic parameters; however, they were found to be associated with accumulation in tissues after acute exposure, thereby limiting their use for repeated dose medication.

Development of amorphous form of pharmaceutical compounds represents both an opportunity and a necessity in pharmaceutical development. The opportunity arises from the potential of the amorphous form to improve the pharmacokinetic properties and bioavailability rather than the corresponding crystalline form.

The compound **1** was made into amorphous form and confirmed through XRD spectra (◘ **Fig. 3a**). The amorphous form of compound **1** was evaluated for its pharmacokinetic properties and its efficacy was evaluated using sucrose (5% w/v) consumption model in Zucker fa/fa rats.

The results are provided in ◘ **Tables 1 and 2**:

The crystalline form of the compound **1** showed significant appetite suppression in rodent model (◘ **Table 1**), however its pharmacokinetic profile was poor (◘ **Table 2**).

The in vivo efficacy study of the amorphous form of compound **1** in 5% sucrose solution intake model in female Zucker fa/fa rats at single oral dose of 10 mg/kg (◘ **Table 1**) demonstrates better reduction in the sucrose solution consumption than the corresponding crystalline form.

The pharmacokinetic parameters of both the crystalline and amorphous form of compound **1** were evaluated (◘ **Table 2**). The

plasma concentration C_{max} at AUC exposure of the amorphous form of the compound **1** is significantly improved and better than the C_{max} of the corresponding crystalline form of the compound **1**. Further, compound **1** exhibited acceptable safety profile upto a dose 30 times of the efficacy dose. No changes in body weight and weights of the organs (kidney, heart and liver) were observed upto 300 mg/kg dose in a 14 day repeat dose study. The compound also did not bind to hERG upto 3 micromolar concentrations.

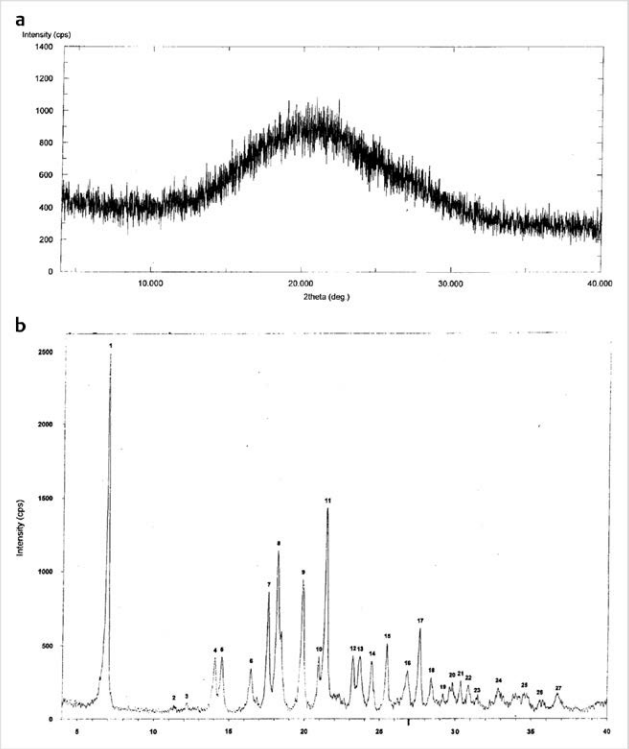

Fig. 3 XRD patterns of amorphous form **a** and crystalline form **b** of compound **1**.

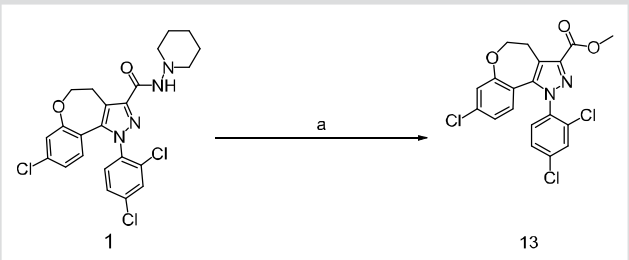

Fig. 4 Structure of compound **13**. Reagents and conditions: **a** acetone, heat at 40 °C till clear solution, HCl.

Based on the impressive in vitro, in vivo and pharmacokinetic parameters of the amorphous form of the compound **1** as a possible neutral CB1 antagonist, the compound has been selected for further development.

In order to prepare sufficient quantities of the compound **1** to support the development program a novel process for preparation of the compound **1** was developed as described in **Fig. 2**. The process described in **Fig. 2** is scalable and gives the compound **1** in high yield (77.6 %).

Conclusion

Early process development, synthesis and identification of the amorphous form of the potent CB1 antagonist compound **1** is described, The improved process described herein is scalable, gives the stable amorphous form in high yield and is suitable for meeting the needs of long term development. The compound **1** demonstrated favourable safety profile in rodent models. Initial pharmacological data of compound **1** are indicative that the compound may be a neutral antagonist and it may not be accompanied by behavioral signs of nausea etc. during the feeding suppression induced by compound **1.** Additional research will be necessary to confirm this hypothesis.

Experimental Section

Synthesis

Synthetic materials and methods

Reagents and solvents were obtained from commercial suppliers and used without further purification. Flash chromatography was performed using commercial silica gel (230–400 mesh). Melting points were determined on a capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Shimadzu FT IR 8300 spectrophotometer (Vmax in cm^{-1}, using KBr pellets or Nujol). The ^1H NMR spectra were recorded on a Brucker Avance-300 spectrometer (300MHz). The chemical shifts (δ) are reported in parts per million (ppm) relative to TMS, either in $CDCl_3$ or DMSO-d_6 solution. Signal multiplicities are represented by s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), bs (broad singlet), and m (multiplet). ^{13}CNMR spectra were recorded on Brucker Avance-400 at 100MHz either in $CDCl_3$ or DMSO-d_6 solution. Mass spectra (ESI-MS) were obtained on Shimadzu LC-MS 2010-A spectrometer. HPLC analysis were carried out at λ_{max} 220 nm using column ODS C-18, 150nm×4.6nm×4µ on AGILENT 1100 series.

8-Chloro-3,4-dihydro-2H-benzo[b]oxepin-5-one(6): 4-(3-chlorophenyl)-butanoic acid (128.0g, 596.74mmol) was taken in a round bottom flask and anhydrous dichloromethane (1L) was

Compound	in-vivo (in 5% sucrose solution intake)		
	Total consumption in 4h	% Change vs. Control (Sucrose intake)	5% Change vs. Control (per 100g body wt.)
Control	48.00±2.20		
Rimonabant	8.4±3.3	−66.4±11.0	−62.0±15.3
Crystalline Form of 1	32.2±6.4	−38.7±12.2	−30.2±16.4
Amorphous Form of 1	31.9±5.2	−54.7±11.5	−24.2±15.3

[a] Values indicate Mean±SEM for n=6 in 4 h

Table 1 In vivo efficacy of the crystalline and amorphous form of the compound 4 in 5% sucrose solution intake model in female Zucker fa/fa rats at single oral dose of 10 mg/kg.[a]

Table 2 Mean pharmacokinetic parameters of the crystalline and amorphous form of the 1 in fasted female Zucker fa/fa rats p.o. at 10 mg/kg.

Compd.	Route	dose (mg/kg)	T_{max} (h)	C_{max} (*n*g/mL)	$T_{1/2}$ (h)	AUC(0–∞) (h.*n*g/mL)
1 (crystalline)	Oral	10	4.5±0.7	223±13	23.9±6.8	3241±125
1 (Amorphous)		10	4.2±1.2	575±32	22.3±2.9	6338±234

[a] Values indicate mean±SD for n=6

added to it. The solution was stirred and cooled to −20°C. To this solution oxalyl chloride (74.06 mL, 835.43 mmol, 1.4 eq) was added drop wise at −20°C over a period of 15–20 min. The resulting solution was stirred at 0°C for 30 min and 26–28°C for 1.5 h. The progress of the reaction was monitored by TLC until all starting material was consumed. The reaction was quenched with ethanol and TLC was checked using mobile phase 5% methanol in chloroform. The solvents were removed on a rotatory evaporator under reduced pressure to afford brown oil. Separately, in a 4-necked round bottomed flask, anhydrous $AlCl_3$ (95.6 g, 716.08 mmol, 1.2 eq) was taken and to it was added anhydrous dichloromethane (1 L). The suspension was stirred and cooled to 0–5°C. To this cooled suspension, a solution of acid chloride obtained above in anhydrous CH_2Cl_2 (200 mL) was added dropwise at 0–5°C over a period of 20–25 min. The resulting solution was stirred at 26–27°C for 30 min. The progress of the reaction was monitored by TLC using mobile phase 5% methanol in chloroform. The reaction mixture was poured into mixture of demineralized water (DM water) and crushed ice (3 L) in 5L round bottom flask followed by CH_2Cl_2 (1 L). The mixture was stirred at 26–27°C for 16 h. The organic layer was separated, washed with DM water (3×1 L). The organic layer was separated, dried over anhydrous Na_2SO_4 and treated with activated charcoal (3-tea spoon) at 38–39°C for 15–20 min and filtered hot through Hyflow. The solvents were removed on a rotatory evaporator under reduced pressure to yield a brown oil (118 g, 100%).

IR (KBr cm^{-1}): 2970, 2887, 1685, 1595, 1087, 821, 767; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.70 (dd, J=8.72 Hz, J=0.77, 1H), 7.06 (m, 2H), 4.25 (t, J=6.60 Hz, 2H), 2.88 (t, J=6.97 Hz, 2H), 2.22 (m, 2H); ESI-MS m/z (Relative intensities) (+ve mode) 256.8 $(M+K)^+$.

(8-Chloro-5-oxo-2,3,4,5-tetrahydro-benzo[b]oxepin-4-yl)-oxo-acetic acid ethyl ester (7): Anhydrous ethanol (1500 mL) was taken in a round bottom flask and small pieces of sodium metal (31.10 g, 1526.71 mmol, and 2.0 eq) were added to it with stirring. The solution was stirred till all Na metal was dissolved. The solution was cooled to 25–27°C. To this diethyl oxalate (103.67 mL, 763.335 mmol, 1.0 eq) was added drop wise at 25–27°C over a period of 15–20 min and stirred for 25–30 min. at the same temperature. To this was added a solution 8-Chloro-3,4-dihydro-2H-benzo[b]oxepin-5-one (150.0 g, 763.335 mmol) in ethanol (2000 mL) drop wise at 25–27°C over a period of 15–20 min. The resulting yellow solution was stirred at 25–26°C for 3–4 h and the progress of reaction was monitored by TLC using mobile phase 10% ethyl acetate in petroleum ether. The reaction mixture was diluted with DM water (2500 mL), acidified with 2 N HCl to pH 4 and extracted with $CHCl_3$. The chloroform layer was separated, washed with DM water, dried over anhydrous Na_2SO_4 and evaporated on a rotatory evaporator under reduced pressure to get yellow oil, which solidifies upon standing. The solid was slurried in diethyl ether (1 L) and filtered to get yellow solid (110 g, 48.6%).

IR (KBr cm^{-1}) 3417, 3109, 1830, 1714, 1614, 1595, 1544, 1207, 1089, 661, 617, 565, 447; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.93 (d, J=9.3 Hz, 1H), 7.26 (m, 2H), 4.37 (m, 2H), 2.88 (m, 2H).

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid ethyl ester (8): (8-Chloro-5-oxo-2,3,4,5-tetrahydro-benzo[b]oxepin-4-yl)-oxo-acetic acid ethyl ester (22.0 g, 74.199 mmol) **7** was taken in a round bottom flask and to it was added anhydrous ethanol (225 mL). The suspension was stirred and cooled to 5–7°C. To this suspension, 2,4-Dichlorophenyl hydrazine hydrochloride (17.90 g, 83.845 mmol, 1.13 eq) was added portion wise at 5–7°C. The resulting suspension was stirred at 5–7°C for 15–20 min and brought to 25–26°C. Isopropyl alcohol: HCl (2.2 mL) was added to this mixture and refluxed at 75–77°C for 2 h. The progress of the reaction was monitored by TLC using mobile phase 20% ethyl acetate in petroleum ether. The reaction mixture was cooled to 25–27°C and the solid separated out was filtered on a Buchner funnel under reduced pressure and dried to afford orange solid (38.0 g, 100%).

IR (KBr cm^{-1}) 3425, 1724, 1679, 1564, 1535, 1454, 1083, 906, 813; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.40 (t, 1H), 7.34 (d, J=2.25 Hz, 2H), 6.83 (dd, J=8.58 Hz, 2H), 6.66 (d, J=8.58 Hz, 1H), 4.50-4.31 (m, 4H), 3.46 (d, 4H), 1.44 (t, 3H); ESI-MS m/z (Relative intensities) (+ve mode) 458.5 $(M+H)^+$.

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylicacid(9): 8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid ethyl ester (38.0 g, 86.857 mmol) **(8)** was taken in a round bottom flask and to it was added methanol (300 mL). To this, a solution of KOH (9.73 g, 173.174 mmol, 2.0 eq) in methanol: water (1:1, 200 mL) was added and reaction mixture was refluxed at 65–68°C for 2.0 h. The progress of the reaction was monitored by TLC using 10% methanol in chloroform as mobile phase. The reaction mixture was cooled to 25–26°C, poured into ice cold water and acidified to pH 4 using 10% HCl solution. The solid separated out was filtered on a Buchner funnel under suction, washed with water, dried under suction. The solid was further taken in isopropyl alcohol (IPA), stirred for 10–15 min and filtered to afford an off white solid (20.2 g, 56.8%).

IR (KBr cm^{-1}) 3450, 3082, 1687, 1596, 1568, 1431, 1386, 1035, 985, 567, 549, 455; ^1H NMR (400 MHz, DMSO-d_6) δ (ppm) 13.08 (bs, 1H), 7.88 (d, J=2.04 Hz, 1H), 7.72-7.64 (m, 2H), 7.23 (d, J=2.13 Hz, 1H), 7.01 (dd, J=2.19 Hz, J=8.58 Hz, 1H), 6.71 (d, J=8.58 Hz, 1H), 4.04 (m, 4H); ESI-MS m/z (Relative intensities) (+ve mode) 410.5 $(M+H)^+$.

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid piperidin-1-ylamide (1), crystalline: 8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid (5.0 g, 12.25 mmol) was taken in a round bottom flask and toluene (30 mL) was added to it. To this solution, thionyl chloride (2.91 g,

24.5mmol) was added and the mixture was refluxed at 107–108 °C for 30 min. The complete conversion of acid to acid chloride was confirmed by TLC. Subsequently, the reaction mixture was cooled to 30–35 °C and transferred into single neck round bottom flask. The solvents were evaporated on a rotatory evaporator under reduced pressure to afford an oil. The oil was further diluted in anhydrous CH_2Cl_2 (20 mL), cooled to 0–5 °C in an ice bath and treated with 1-amino piperidine (1.83 g, 18.38 mmol). The resulting mixture was stirred at 25–26 °C for 15–20 min. The progress of the reaction was monitored by TLC using mobile phase 50% EtOAc in hexane. The reaction mixture was diluted with DM water (200 mL) and extracted with toluene. The organic layer was separated, washed with DM water (100 mL), brine solution (100 mL), dried over anhydrous Na_2SO_4 and solvents were removed on a rotatory evaporator under reduced pressure to afford an oil. The oil was diluted with anhydrous methanol (25 mL), cooled to 0–5 °C in an ice bath and treated with ethereal HCl (5–6 mL). The solvents were evaporated on a rotatory evaporator under reduced pressure to afford brown solid. The crude solid was triturated in ethyl acetate, filtered on a Buchner funnel under suction and dried to afford an off white solid (5.0 g, 77.6%).
^{1}HNMR: ($CDCl_3$, 300MHz): δ 10.35 (s, 1H), 7.51 (d, J=1.74Hz, 1H), 7.40 (m, 2H), 7.34 (m, 1H), 7.14 (d, J=1.95Hz, 1H), 6.62 (d, J=8.55Hz, 1H), 4.38 (m, 2H), 4.06 (bs, 3H), 3.48 (bs, 3H), 3.26 (m, 2H), 3.11 (m, 2H), 1.67(bs, 4H), 1.41(m, 1H), 1.18 (m, 1H);
^{1}HNMR: (DMSO-D_6, 300MHz): δ 10.53 (s, 1H), 7.89 (d, J=2.01Hz, 1H), 7.78 (d, J=8.49Hz, 1H), 7.68 (dd, J=10.47, 1.95Hz, 1H), 7.26 (d, J=1.95Hz, 1H), 7.03 (dd, J=10.56, 1.95Hz, 1H), 6.72 (d, J=8.61Hz, 1H), 4.45 (bs, 1H), 4.25 (bs, 1H), 3.23 (bs, 4H), 3.06 (m, 3H), 1.72 (bs, 4H), 1.42(bs, 2H), 1.16(t, 1H), 0.98 (t, 1H).
The basification of the HCl salt of compound **1** with NaOH gave the compound of **1** in the crystalline form.
^{1}HNMR: ($CDCl_3$, 300MHz) δ 7.8 (d, J=7.5Hz, 2H), 7.4 (d, J=7.5Hz, 2H), 7.39 (d, J=8.4Hz, 1H), 7.34 (d, J=1.8Hz, 1H), 7.3 (m, 2H), 7.0 (d, J=8.4Hz, 1H), 6.5 (d, J=5.4Hz, 1H), 3.27 (d, J=5.1Hz, 2H), 3.18 (d, J=5.1Hz, 1H), 2.6 (m, 3H), 1.5 (s, 3H); Melting point (by DSC): Onset=195.3 °C, peak=196.6°C.
XPRD: 6.97, 16.42, 17.53, 18.19, 18.35, 19.8, 20.93, 21.4, 23.69, 27.62°±0.2° degrees 2θ.

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid piperidin-1-ylamide (1), dimethyl formamide solvate (11): Placed crystalline compound **1** (0.50g, 1.017mmol) into round bottom flask, followed by addition of dimethyl formamide (15 mL). The solution was heated to 60–70 °C for 2 h to obtain a clear solution. The solution was cooled to 27–30 °C for 48 h whereby white crystals were obtained.
Weight of White solid substance=0.258g
IR (KBr cm^{-1}) 3412, 2941, 1678, 1595, 1564, 1492, 1290, 1265, 1209, 1153, 1026, 983, 956, 869, 817, 810;
1H NMR (400MHz, $CDCl_3$) δ (ppm) 8.01 (s, 1H), 7.63 (s, 1H), 7.53 (d, J=2.4Hz, 1H), 7.39 (dd, J=8.6Hz, J=2.0Hz, 1H), 7.33 (dd, J=9.2Hz, J=4.4Hz, 1H), 7.14 (d, J=2.0Hz, 1H), 6.79 (dd, J=8.8Hz, J=2.0Hz, 1H), 6.60 (d, J=8.4Hz, 1H), 4.38 (bs, 2H), 3.49 (bs, 2H), 2.95 (s, 3H), 2.85(s, 3H), 2.95-2.85 (m, 4H), 1.78-1.72 (m, 4H), 1.45-1.43 (m, 2H).
ESI-MS m/z (Relative intensities) (+ve mode) 493.0 $(M+H)^+$ (100%).

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid piperidin-1-ylamide oxalate {12 b}: Compound **1** (0.500 g (1.017 mmol) was taken in a round bottomed flask, followed by 10 mL acetone. The suspension was warmed upto 45–50 °C to get clear solution. To the solution of **1** in acetone a solution of oxalic acid (0.075 g, 1.017 mmol) in 3 mL acetone was added slowly. The resulting clear solution was cooled to 0–5 °C and stirred for 35–40 min. The solid separated out which was filtered, washed with acetone and dried under vacuum to afford white solid (0.267 g, 45.9%).
IR (KBr cm^{-1}) 3412, 2951, 1899, 1691, 1597, 1564, 1492, 1236, 817, 704;
^{1}H NMR (400MHz, DMSO-d_6) δ (ppm) 9.19 (s, 1H), 7.90 (d, J=2.4Hz, 1H), 7.80 (d, J=8.4Hz, 1H), 7.71 (dd, J=8.4Hz, J=2.4Hz, 1H), 7.27 (d, J=2.4Hz, 1H), 7.04 (dd, J=8.6Hz, J=2.4Hz, 1H), 6.74 (d, J=8.4Hz, 1H), 4.49 (bs, 1H), 4.26 (bs, 1H), 3.40 (bs, 2H), 3.28 (bs, 2H), 2.82-2.80 (m, 4H), 1.64-1.58 (m, 4H), 1.4-1.3 (m, 2H).
ESI-MS m/z (Relative intensities) (+ve mode) 493.05 (M+H)+ (80%).

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid piperidin-1-ylamide benzene sulfonate salt {12c}: Placed **1** (0.500 g, 1.017 mmol) in a round bottomed flask and to it was added acetone (10 mL). The suspension was warmed upto 45–50 °C to get clear solution. To the solution of **1** in acetone, benzene sulfonic acid (0.160 g, 1.017 mmol) in acetone (3 mL) was added slowly. The resulting clear solution was cooled to 0–5 °C and stirred for 35–40 min. The solid separated out was filtered, washed with acetone and dried under vacuum to afford a white solid (0.350 g, 53%).
IR (KBr cm^{-1}) 3383, 3153, 2945, 1896, 1701, 1697, 1606, 1552, 1219, 1151, 981, 956, 896, 731; ^{1}H NMR (400MHz, DMSO-d_6) δ (ppm) 11.05 (bs, 1H), 7.94 (d, J=2.4Hz, 1H), 7.83 (d, J=8.4Hz, 1H), 7.73 (dd, J=8.6Hz, J=2.4Hz, 1H), 7.65-7.61 (m, 2H), 7.37-7.30 (m, 4H), 7.07 (dd, J=8.6Hz, J=2.4Hz, 1H), 6.77 (d, J=8.8Hz, 1H), 4.55-4.52 (m, 1H), 4.30-4.29 (m, 1H), 3.46-3.43 (m, 1H), 3.33-3.32 (m, 5H), 1.83-1.80 (m, 4H), 1.5-1.4 (m, 2H). ESI-MS m/z (Relative intensities) (+ve mode) 493.0 $(M+H)^+$ (100%).

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid piperidin-1-ylamide bisulfate {12d}: Placed **1** (1.000 g, 2.034 mmol) in a round bottomed flask and to it was added acetone (15 mL). The suspension was warmed upto 45–50 °C to get clear solution. To the solution of **1** in acetone was added conc. H_2SO_4 (0.108 mL, 2.034 mmol) dropwise. The resulting clear solution was cooled to 0–5 °C and stirred for 15–20 min. The solid separated was filtered, washed with acetone and dried under vacuum to afford a White solid (1.03 g, 85.87%) IR (KBr cm^{-1}) 3404, 3153, 2951, 1708, 1599, 1550, 1369, 1215, 1049, 987, 958, 868, 812;
^{1}H NMR (400MHz, DMSO-d_6) δ (ppm) 10.85 (bs, 1H), 7.93 (d, J=2.4Hz, 1H), 7.83 (d, J=8.8Hz, 1H), 7.71 (dd, J=8.6Hz, J=2.4Hz, 1H), 7.29 (d, J=2.4Hz, 1H), 7.07 (dd, J=8.6Hz, J=2.4Hz, 1H), 6.76 (d, J=8.8Hz, 1H), 4.55-4.52 (m, 1H), 4.29-4.28 (m, 1H), 3.45-3.3.42 (m, 1H), 3.33-3.31 (m, 1H), 3.35-3.15 (m, 4H), 1.80-1.77 (m, 4H), 1.5-1.4 (m, 2H). ESI-MS m/z (Relative intensities) (+ve mode) 493.0 $(M+H)^+$ (99%).

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid piperidin-1-ylamide methyl iodide {13e}: Placed **1** (5.000 g, 10.17 mmol) in a round bottomed flask and to it was added acetone (15 mL). The suspension was warmed to 45–50 ° to get clear solution. To this solution methyl iodide (14.435 g, 6.322 mmol) was added. The resulting clear solution was refluxed over a period of 16h. The solid precipitated was dissolved in distilled acetone and resulting solution was concentrated under reduced pressure at 45 °C to get a yellow solid. The solid was filtered, washed with acetone and dried under vacuum to yield off-white solid (5.3 g, 82.81%) IR (KBr cm^{-1}) 3419, 2958, 1593, 1564, 1496, 1309, 1294, 1209, 1030, 954, 920, 869, 812; 1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.83 (d, J=2.4 Hz, 1H), 7.62 (dd, J=8.6 Hz, J=2.0 Hz, 1H), 7.57 (d, J=8.8 Hz, 1H), 7.18 (d, J=2.0 Hz, 1H), 6.96 (dd, J=8.8 Hz, J=2.0 Hz, 1H), 6.66 (d, J=8.4 Hz, 1H), 4.40 (bs, 1H), 4.22 (bs, 1H), 4.12 (bs, 2H), 3.41 (s, 3H), 3.22-3.14 (m, 4H), 2.01-1.98 (m, 2H), 1.66-1.58 (m, 3H), 1.42-1.36 (m, 1H). ESI-MS m/z (Relative intensities) (+ve mode) 507.0 $(M+H)^+$ (100%).

8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-1,2-diaza-benzo[e]azulene-3-carboxylic acid piperidin-1-ylamide (1), amorphous: The crystalline 8-Chloro-1-(2,4-dichloro-phenyl)-4,5-dihydro-1H-6-oxa-diaza-benzo[c]azulene-3-carboxylic acid-piperidin-1-ylamide (5 g, 10.17 mmol) was placed in a round bottom flask and added of dichloromethane (100 mL). The resulting solution was stirred at 27–29°C for 10 min. The solvents were removed under reduced pressure at 50°C to afford white solid. The solid obtained was analyzed by XRD pattern, established it to be in the amorphous form. The DSC thermogram showed 2 peaks one at 182.9°C and the other at 196.3°C.

In vitro cAMP assay

Fatty acid-free BSA, IBMX (isobutyl methyl xanthine), RO20-1724 {4-[(3-butoxy-4-methoxyphenyl) methyl]-2-imidazololidinone}, forskolin and DMSO (hybrimax) were purchased from Sigma Chemical Co. cAMP detection ELISA kit was from Assay Designs, USA. Tissue culture reagents were purchased from Sigma and Hi-media. Other reagents used were all of analytical grade. The cAMP assay was carried out in Chinese Hamster Ovarian (CHO) cells (CHOK1) stably expressing human CB1 receptor following the method of Rinaldi-Carmona et al [31]. Cells grown to 80% confluence were maintained in HAM'S F12 medium containing 10% heat inactivated dialyzed fetal bovine serum and 0.8 mg/mL G-418. Cells were seeded at a density of 50000 cells/well in 24-well plate, grown for 16–18 h, washed once with PBS and incubated for 30 min at 37°C in plain HAM'SF12 containing 0.25% free fatty acid BSA, IBMX (0.1 mM) and RO20-1724 (0.1 mM). IBMX, the pan phosphodiesterase inhibitor and RO20-1724, the specific phosphodiesterase-4 inhibitor were added to restore cAMP up to the detection limit. After 5 min incubation with the drugs, forskolin was added at a final concentration of 10 mM and incubation was carried out for another 20 min at 37°C. The reaction was terminated by washing once with PBS and adding 200 lL lysis buffer comprising 0.1 N HCl and 0.1% Trition X-100. The lysates were centrifuged and aliquotes from supernatants were used for detection of cAMP by ELISA as per the manufacturer's protocol.

5% Sucrose solution intake in Zucker fa/fa rats

All the animals used in the study were procured from the Animal Breeding Facility of Zydus Research Center. Institutional Animal Ethical Committee approved all the study protocols. Female Zucker fa/fa rats (age of 10–12 weeks and 300–350 g of weight) were used for in vivo experiments. Compounds were suspended with 0.5% carboxymethyl cellulose sodium salt in distilled water. The test compounds were administered at the dose of 10 mg/kg and by oral route in a volume of 2 mL/kg body weight. The obese Zucker fa/fa rats were housed individually and subjected to training for consuming 5% sucrose solution over a period of 4 h, by allowing access to the 5% sucrose solution in the bottles. Food and water were withdrawn during this time. This training was given for 6 consecutive days, at the same time of the day. On seventh day, the animals were randomized into groups of 6 animals each and treated with the test compounds. After 1 h of treatment, the animals were exposed to the 5% sucrose solution for 4 h as that of the training schedule. The amount of sucrose solution consumed by each animal was calculated. Difference between the control and treatment groups were analyzed by performing one way ANOVA followed by Dunnett's test on sucrose solution consumption using Graph pad Prism software.

Pharmacokinetics experiment

Pharmacokinetics of the test compound was studied *via* per-oral route of administration in *Zucker fa/fa rats* of 8–10 weeks of age (6 animals). Animals were fasted for 18 h and food was supplied after 4 h of administration of the test compound. There was free access to water throughout the study. A homogenous suspension of the test substance was prepared in 0.5% w/v CMC in normal saline and a per-oral dose of 10 mg/kg was administered. After the administration of the test compounds, blood samples were withdrawn at various time intervals through retro-orbital plexus and collected into heparinized micro centrifuge tubes. Plasma was separated by centrifugation at 4000 rpm for 5 min at ambient temperature and analyzed immediately. Remaining samples were stored at −20°C until analyzed.

Analysis was carried out by taking an aliquots of 180 μL plasma and 20 μL of internal standard (Atorvastatin) and was extracted with 2.5 mL of extracting solvent (ethyl acetate: acetonitrile 80:20, v/v) in glass test-tube by vortexing with spinix vortex mixture for a minute. This was then centrifuged at 2000 rpm for 2.0 min. The supernatant was transferred to another glass test-tube and the solvent was evaporated under nitrogen using Zymark evaporator at 40°C. Finally, the tubes were reconstituted with 0.1 mL diluent (acetonitrile: methanol: water 40:40:20, v/v/v). The reconstituted samples were analyzed on Agilent 1100 Series HPLC system with a mobile phase of 0.05% v/v trifluoroacetic acid in water: acetonitrile (32:68, v/v); flowing at a flow rate of 1.0 mL/min through a Kromasil 250 mm×4.6 mm×5 μ column maintained at 30°C. Chromatographic separation was achieved within 15 min. Agilent software version Chemstation Rev.A.09.01. (1206) was used to acquire and process all chromatographic data. Quantification was based on a series of calibrators ranging from 0.031 to 32 μg/mL, prepared by adding test compound to drug free rat plasma. Quality control samples were analyzed in parallel to verify that the system performs in control. Pharmacokinetic parameters namely; maximum plasma concentration (C_{max}), time point of maximum plasma concentration (t_{max}), area under the plasma concentration−time curve from 0h to infinity ($AUC_{0-\infty}$) and half-life of drug elimination during the terminal phase ($t_{1/2}$) were calculated from plasma concentration vs. time data, by standard non-compartmental

methods, using the WinNonLin software version 4.0.1 procured from Pharsight Corporation, USA.

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Conflict of Interest

None

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