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Design, Synthesis And Chemical Stability Of Indolizine Derivatives For Antihypertensive Activity.

Basavaraj M[1]*, Giles D[1], Das Ak[2], Suresh J[1], and Ganesh S Andhale[3].

[1]Department of Pharmaceutical Chemistry, Nitte College of Pharmaceutical Sciences, Yelahanka, Bengaluru, Karnataka, India.
[1]Department of Pharmaceutical chemistry, Acharya & BM Reddy College of Pharmacy, Bengaluru, Karnataka, India
[2]Department of Pharmaceutical Chemistry, Krupanidhi College of Pharmacy, Bengaluru, Karnataka, India
[3]Department of Pharmaceutical Chemistry, Alard College of Pharmacy, Pune, India

ABSTRACT

In the present work we have synthesized the substituted 2- Phenyl indolizine (**1a-f**) by the reaction of 2-methylpyridine with α-haloaryl ketones produced substituted 2-phenyl indolizine. In the next step 2-substituted indolizine undergo Vilsmeyer reaction to produce substituted 2-phenylindolizine-3-carbaldehyde and this treated with hydralazine to form the derivatives (**3a-f**). Synthesized compounds were subjected to chemical hydrolysis at pH 1.2, 6.8 and 7.4 and all the compounds have shown significant stability. IR, NMR and Mass spectroscopy techniques used to confirm the structure of final derivatives. All the synthesized compounds were tested for antihypertensive activity by non-invasive blood pressure (NIBP) measurements (tail-cuff method) in rats. Almost all the tested compounds displayed considerable decrease in the blood pressure as compared to control. Compounds showed significant antihypertensive activity comparable to the standard drug hydralazine.

Keywords: Indolizine, prodrugs, antihypertensive activity, hydralazine

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Corresponding author

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INTRODUCTION

Hypertension remains a major health problem in most countries because of its impact on the mortality and morbidity of patients. Indeed, hypertension accounts for more than 5.8% of total deaths, 1.9% of years of life lost and 1.4% disability adjusted life years all over the world.[1] Hypertension affects an estimated 1 billion people worldwide and this number is expected to increase to 1.56 billion people by the year 2025.[2] Therefore hypertension prevention and control is a challenge.

Most of the drugs for cardiovascular diseases have low oral bioavailability, short duration of action, first pass metabolism and variable lipophilicities. Out of the need to overcome these limitations, various prodrugs have been designed for antihypertensive agents.[3]

Hydralazine remained as second line therapy for patients with essential hypertension. Due to its potential to induce angina and tachycardia when used alone, hydralazine is often combined with β–blockers and diuretics. The precise mechanisms underlying the vasorelaxant properties of hydralazine remain unclear, although interference with inositol triphosphate-induced calcium release in arterial smooth muscle cells is a commonly-invoked explanation.[4, 5]

Since prodrugs undergo a chemical reaction to form the parent drug once inside the body, this makes them very effective in controlling the release of a variety of compounds to targeted site and overcome barriers such as poor solubility, permeability and resistance to fast degradation.

In this paper, we report for the chemically stable hydralazine derivatives to be used as prodrugs of hydralazine and screened for the antihypertensive activity.

MATERIALS AND METHODS

Hydralazine obtained as gift samples from Strides Arcolab Bangalore (India). Chemicals used for the synthesis, were purchased from Spectrochem. Solvents were re-distilled before use. Characterization of synthesized derivatives including intermediates, were done by FTIR (Bruker spectrophotometer) and, NMR (^{1}H-NMR and ^{13}C-NMR Spectra Bruker-at 400MHz, 500 MHz spectrometer). Chemical shifts were measured in δ ppm using TMS as internal standard. Mass spectra were recorded on Agilent LC-MS spectrometer. TLC was performed on pre-coated silica gel plates (Merck 60 F_{254}) to check the progress of reaction as well as purity of the synthesized molecules. Melting point was checked by open capillary tube method and reported uncorrected.

General procedure for the synthesis of Substituted 2- Phenyl Indolizine (**1a-f**):

Substituted Phenacyl bromide (1.146 mmol) was added to a solution of 2-methylpyridine (1.146 mmol) in dry ethanol (25 mL) and the spontaneous reaction was controlled by external cooling. Next day hard mass was broken and washed with dry alcohol and recrystallized from the alcohol to obtain substituted phenacylpicoliniumbromide then it was dissolved in water, sodium bicarbonate added and mixture was boiled for 1 h. The mixture was cooled and the solid was collected and recrystallization done from ethanol.
*2.2*a Phenyl Indolizine (**1a)**: white solid; mp: 201-205 °C. FTIR (KBr, υcm^{-1}): 3196.35(CH, Ar-str), 1326.24 (CN, str); ^{1}H NMR (400 MHZ, $CDCl_3$) *δ* (ppm): 8.207-8.210 (d, 1H, Ar-H), 7.940-7.944 (d, 2H, Ar-H), 7.769-7.798 (m, 2H, Ar-H), 7.356-7.403 (m, 2H, Ar-H), 7.201-7.244 (m, 1H, Ar-H), 6.670-6.693 (m, 1H, Ar-H), 6.074 (s, 1H), 6.500-6.516 (t, 1H, Ar-H); MS: (m/z) 193 (M^+).

2.2b 2-(4-Chlorophenyl) indolizine **(1b)**: pale white solid; mp: 210-215 °C. FTIR (KBr, υcm^{-1}): 2996.12 (CH, str), 1335.16 (CN, str); ^{1}H NMR (400 MHZ, $CDCl_3$) *δ* (ppm)*:* 8.167-8.183 (d, 1H, Ar-H), 7.849-7.852 (d, 1H, Ar-H), 7.595-7.617 (d, 2H, Ar-H), 7.336-7.359 (d, 1H, Ar-H), 6.941-6.962 (d, 2H, Ar-H), 6.634-6.646 (m, 1H, Ar-H), 6.071 (s, 1H), 6.491-6.508 (t, 1H, Ar-H); MS: (m/z) 227 (M^+).

2.2c 2-(4-Bromophenyl) indolizine (**1c**): mp: white solid; 190-195°C. FTIR (KBr, υcm^{-1}): 3103.87 (CH, Ar-str), 1371.14 (CN, str); ^{1}H NMR (400 MHZ, $CDCl_3$): *δ* (ppm): 8.165-8.181 (d, 1H, Ar-H), 7.845-7.850 (d, 1H, Ar-H), 7.591-7.613 (d, 2H, Ar-H), 7.333-7.356 (d, 1H, Ar-H), 6.942-6.965 (d, 2H, Ar-H), 6.635-6.653 (m, 1H, Ar-H), 6.070 (s, 1H), 6.492-6.510 (t, 1H, Ar-H); MS: (m/z) 271 (M^+).

2.2d 2-(4-Fluorophenyl) indolizine (**1d)**: White solid; mp: 195-199°C. FTIR (KBr, υ cm^{-1}): 3117.54(CH, Ar-str), 1378.83 (CN, str);^{1}H NMR (400 MHZ, $CDCl_3$): δ (ppm): 8.162-8.182 (d, 1H, Ar-H), 7.843-7.847 (d, 1H, Ar-H), 7.590-7.611 (d, 2H, Ar-H), 7.331-7.355 (d, 1H, Ar-H), 6.940-6.963 (d, 2H, Ar-H), 6.631-6.651 (m, 1H, Ar-H),6.075 (s, 1H), 6.494-6.514 (t, 1H, Ar-H); MS: (m/z) 211 (M^+).

2.2e 2-(4-Nitrophenyl) indolizine (**1e**): Yellow solid; mp: 214-218 °C. FTIR (KBr, υcm^{-1}): 3102.57 (CH, Ar-str), 1371.20 (CN, str), 1617.59 (NO_2); ^{1}H NMR (400 MHZ, DMSO): δ (ppm):8.152-8.172 (d, 1H, Ar-H), 7.841-7.845 (d, 1H, Ar-H), 7.591-7.612 (d, 2H, Ar-H), 7.333-7.357 (d, 1H, Ar-H), 6.942-6.965 (d, 2H, Ar-H), 6.633-6.653 (m, 1H, Ar-H), 6.081 (s, 1H), 6.496-6.517 (t, 1H, Ar-H); MS: (m/z) 238 (M^+).

2.2f 2-(4-Methylphenyl) indolizine (**1f)**: Green solid; mp: 195-200°C. FTIR (KBr, υcm^{-1}): 3133.12 (CH, Ar-str), 2938.36 (CH, Ali-str), 1326.12 (CN, str);^{1}H NMR (400 MHZ, DMSO): δ (ppm): 8.171-8.188 (d, 1H, Ar-H), 7.894-7.896 (d, 1H, Ar-H), 7.561-7.581 (d, 2H, Ar-H), 7.346-7.368 (d, 1H, Ar-H), 7.176-7.196 (d, 2H, Ar-H), 6.640-6.700 (m, 1H, Ar-H), 6.079 (s, 1H), 6.484-6.500 (t, 1H, Ar-H), 2.300 (s, 3H, CH_3); MS: (m/z) 207 (M^+).

3 General procedure for formylation of 2- Substituted indolizine

An ice cold solution of phosphoryl chloride (0.32 mL, 4.05 mmol) in dimethyl formamide (1mL) was added dropwise to a stirred ice-cold solution of 2-substituted indolizine (**1a-f)** (4.00 mmol) in dimethylformamide (1 mL).The reaction continued for stirring 4 h monitored by TLC and reaction mixture poured into ice cold water and then made strongly basic with sodium hydroxide solution (20%). The resulting precipitate washed with water and dried. Recrystallization done from ethanol and chloroform mixture.

General Method for the preparation of 2-phenyl (4-substituted) indolizin-3-yl) methylene) hydrazinyl) pthalazine

An equimolar quantity of (**2a**, 1mmol) and hydralazine (1mmol) and added 20 mL of ethanol few drops of acetic acid refluxed and reaction was monitored with TLC after completion of reaction the reaction mixture poured into ice cold water and solid formed was filtered and dried, purified with ethanol and chloroform mixture. All the title compounds were synthesized by adopting the same procedure with variation in reaction time.

3a. 1-2-((2-Phenylindolizin-3-yl) methylene hydrazinyl) pthalazine (**3a**): pale white solid; mp: 210-213° C. FTIR (KBr, υcm^{-1}): 3275.16 (NH, str), 3123.58 (Ar. CH, str), 2925.46 (Ali. CH, str), 1627.23 (C=N), 1355.27 (CN, str).^{1}H NMR ($CDCl_3$): δ (ppm): 9.953 (s, 1H, CH), 8.762-8.774 (d, 1H, Ar-H), 8.489-8.507 (1H, d), 7.724-7.838 (m, 5H, Ar-H), 7.448-7.707 (m, 8H, Ar-H), 6.131 (s, 1H, NH). MS: m/z 363.15 (M^+).

1-(2-((2-(4-Chlorophenyl) indolizin-3yl) methylene) hydrazinyl) pthlalzine (**3b**): white solid; mp: 220-225°C. FTIR (KBr, υcm^{-1}): 3278.16 (NH,str), 3115.54 (Ar. CH, str), 2921.16 (Ali. CH, str), 1621.13 (C=N), 1351.27 (CN, str).^{1}H NMR ($CDCl_3$): δ (ppm): 9.923 (s, 1H, CH), 8.761-8.764 (d, 1H, Ar-H), 8.484-8.507 (d, 1H, Ar-H), 7.721-7.831 (m, 4H, Ar-H), 7.428-7.707 (m, 8H, Ar-H), 6.134 (s, 1H, NH). MS: m/z 397.11 (M^+).

1-(2-((2-(4-bromophenyl) indolizin-3yl) methylene) hydrazinyl) pthlalzine (**3c**): pale yellow solid; mp: 231-235° C. FTIR (KBr, υcm^{-1}): 3268.17 (NH, str), 3125.54 (Ar. CH, str), 2922.15 (Ali. CH, str), 1627.15 (C=N), 1341.28 (CN, str). ^{1}H NMR ($CDCl_3$) δ (ppm): 9.892 (s, 1H, CH), 8.751-8.756 (d, 1H, Ar-H), 8.481-8.488 (d, 1H, Ar-H), 7.724-7.829 (m, 4H, Ar-H), 7.428-7.707 (m, 8H, Ar-H), 6.133 (s, 1H, NH). MS: m/z 441.06 (M^+).

1-(2-((2-(4-fluorophenyl) indolizin-3yl) methylene) hydrazinyl) pthalalzine (**3d**): white solid; mp: 231-235° C. FTIR (KBr, υcm^{-1}): 3262.15 (NH, str), 3128.58 (Ar. CH, str), 2928.19 (Ali. CH, str), 1629.19 (C=N), 1347.29 (CN, str). ^{1}H NMR ($CDCl_3$): δ (ppm): 9.821 (s, 1H, CH), 8.749-8.755 (d, 1H, Ar-H), 8.482-8.489 (d, 1H, Ar-H), 7.719-7.829 (m, 4H, Ar-H), 7.421-7.701 (m, 8H, Ar-H), 6.139 (s, 1H, NH). MS: m/z 381.14 (M^+).

1-(2-((2-(4-nitrophenyl) indolizin-3yl) methylene) hydrazinyl) pthalalzine (**3e**): yellow solid; mp: 155-160° C. FTIR (KBr, υcm^{-1}): 3265.18 (NH, str), 3124.61 (Ar. CH, str), 2941.27 (Ali. CH, str), 1624.19 (C=N), 1348.17 (CN, str). ^{1}H NMR ($CDCl_3$): δ (ppm): 9.814 (s, 1H, CH), 8.744-8.750 (d, 1H, Ar-H), 8.481-8.487 (d, 1H, Ar-H), 7.717-7.828 (m, 4H, Ar-H), 7.425-7.702 (m, 8H, Ar-H), 6.138 (s, 1H, NH). MS: m/z 408.13 (M^+).

1-(2-((2-(4-methylphenyl) indolizin-3yl) methylene) hydrazinyl) pthalalzine (**3f**): pale green solid; mp: 235-240° C. FTIR (KBr, υcm^{-1}): 3362.18 (NH, str), 3127.65 (Ar. CH, str), 2948.21 (Ali. CH, str), 1624.17 (C=N), 1347.29 (CN, str). ^{1}H NMR ($CDCl_3$): δ (ppm): 9.815 (s, 1H, CH), 8.749-8.755 (d, 1H, Ar-H), 8.482-8.489 (d, 1H, Ar-H), 7.717-7.821 (m, 4H, Ar-H), 7.421-7.701 (m, 8H, Ar-H), 6.129 (s, 1H, NH), 2.321 (s, 3H, CH_3) MS: m/z 377.14 (M^+).

Chemical Hydrolysis studies

Hydrolytic behavior of synthesized prodrugs was studied in Simulated Gastric Fluid (pH 1.2); Simulated Intestinal Fluid (pH 6.8); Simulated Plasma Fluid (pH 7.4). Chemical hydrolysis studies was carried out with USP-II paddle apparatus at a rotational speed of 50 rpm, temperature of 37±1°C, 900 mL solution of pH 1.2, 6.8 and 7.4 were used as dissolution media. 1 mL of the hydrolysis medium was taken out at 0 minute and every 15 min. for 120 min. 1 mL of the pH solution was added to the dissolution vessel. The sample withdrawn was analyzed with the HPLC using Phenomenex Luna C_{18} column (250 mm x 4.6 mm id, 5 µm particle size), LC solutions software and mobile phase acetonitrile: water 70:30. Flow rate of mobile phase was kept at 1 mL/min at pressure 120-135 psi and UV detector (SPD-20A with D2 lamp) was used and retention time and peak area were noted at 274nm.

Pharmacology

All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of Acharya & B M Reddy College of Pharmacy, Bengaluru, constituted in accordance with the guidelines of the committee for the purpose of control and supervision of experiment on animals (CPCSEA), Government of India.

The investigations were conducted on albino rats weighing 200-250 g of either sex. All the rats were housed in a temperature and humidity controlled room at an ambient temperature of 25± 2 °C with 12 h light/dark cycle and allowed free access to food and water except at the time they were brought out of the cage.

Antihypertensive activity

Antihypertensive activity of synthesized compounds was assessed by Deoxycorticosterone Acetate salt (DOCA-salt) induced hypertension in rats and the non-invasive tail-cuff method was employed to determine systolic blood pressure (SBP) in rats. Hydralazine was used as standard drug.

DOCA-salt hypertension: Hypertension in rats was induced by injecting twice weekly with 20 mg/kg sac. Deoxycortisoneacetate (DOCA) in olive oil for 4 weeks. Drinking water was replaced by 1% w/v NaCl solution. Injection of DOCA-salt was not administered to the control group of rats that received vehicle injections and tap water instead. Blood pressure started to rise after one week and the systolic value reached between 160 and 180 mm Hg after 4 weeks. Animals were divided into three groups. Group-I served as control, group-II received newly synthesized compounds and group-III received standard drug hydralazine.

Statistical Analysis: All data obtained from animal experiments were calculated as mean ± SEM. Statistical differences between the synthesized compounds and the control were tested by one-way ANOVA followed by Dunnetts multiple comparison tests. Level of significance: $P < 0.05$, $P < 0.01$ level, $P<0.0001$.

Non-invasive blood pressure (NIBP) measurements (tail-cuff method)

The blood pressure (BP) was determined with a BIOPAC student, NIBP with computerized BP monitor. This system measures blood pressure (BP) by recording the cuff pressure at which the interrupted blood flow returns to the tail. Training the rats for tail-cuff blood pressure measurements was necessary to reduce the stress associated with the BP measurements and hence reduces the variability of BP with successive measurements. Training consisted of six sessions over 3 days. On day one, rat was introduced into cylindrical restrainer for 5 min. per session. The tail cuff was inflated five times in quick succession. By day three, the training was extended to 10 min. per session. The result of training was to reduce the standard deviation around mean BP. At the end of session rats were ready for BP recording. They were restrained by being placed into cylindrical restrainer. Tail pulse was detected by passage of tail through a narrow tail-cuff sensor attached

to the amplifier. BP measurements were started by automatic inflation of tail-cuff to greater than 200 mmHg and release of pressure. The results were recorded in form of graph. The computer provides two tracings that start and stop at the same time. The lower trace channel plots cuff pressure, which is calibrated at 500 mmHg at full scale. The tracing sharply rises when applied to the tail-cuff and falls off gradually during the 15-20s of the test. The upper trace channel monitors pulse, with fluctuations about the center line suddenly appearing at the onset of pulsations. The first onset of pulse was taken as the systolic blood pressure. Initiation of pulse pressure was determined. When the baseline amplitude increased in accordance to the set maximal inflated cuff pressures, maximal inflation was set at 200 mmHg. Blood pressure recording was considered to be successful if the rat did not move and a clear initial pulse could be seen. Ten tail-cuff measurements were made in a session. The BP for the session was accepted as the average of four BP readings that were within 5 mmHg or the average of 10 readings that were within 8 mmHg. On the first day of experiment the test compounds were administered by oral feeding using an oral feeding needle. The test compounds (selected prodrugs) were prepared in 0.5% carboxymethyl cellulose (CMC) and dose of 20 mg/kg orally. Prior to the dosing the animals, initial graph reading was taken to record the BP. Average readings were calculated by employing ANOVA method.

RESULTS AND DISCUSSION

The target compounds were synthesized as outlined in Scheme 1. 2-substituted Phenyl indolizines synthesized by using substituted phenacyl bromide and was converted to 3-formyl 2- phenyl substituted indolizine by using $POCl_3$ and DMF, which was further treated with hydralazine by using ethanol and few drops of glacial acetic acid to yield corresponding of 2-phenyl(4-substituted) indolizin-3-yl) methylene) hydrazinyl) pthalazine in good yields. The synthesized compounds were characterized by IR, ^1H, NMR, and Mass spectral analysis.

All the reactions were monitored using precoated TLC plates. The absence of TLC spots for starting materials and appearance of single new TLC spot at different R_f value ensured completion of the reaction. The TLC plates were visualized either by iodine vapors or by viewing in UV-visible chamber. The reaction product of all the reactions were purified initially by different workup processes to remove unreacted starting materials if any and then recrystallization was carried out. The prodrugs (**3a-f)** have shown the FTIR spectral data for NH peak in the range of 3275.16-3278.16 cm^{-1}, Ar. C-H peak in the range of 3123.58-3125.54 cm^{-1}, C=N peak in the range of 1621.13 cm^{-1}.

In the ^1H NMR spectra, all protons were seen according to the expected integral values. The ^1H NMR of prodrugs **(3a-f)** have shown the CH singlet peak in the range of δ 9.923-9.953 ppm, Ar. C-H multiplet peak in the range of δ 6.706-8.514 ppm, two singlet peak for N-H group in the range of δ 6.139-6.131 ppm, prodrugs were confirmed through the absence of CHO peak of prodrug and the new peak was observed for CH=N in the spectra of all the prodrugs.

Chemical hydrolysis

The Chemical hydrolysis of the synthesized prodrugs **(3a-f)** were studied to determine the stability of prodrug at pH 1.2 (non enzymatic Simulated Gastric Fluid, SGF), pH 6.8 (non enzymatic Simulated Intestinal Fluid) whereas potential to generate as the hydralazine at physiological pH 7.4 at 37±5°C using HPLC. None of the prodrugs showed hydrolysis in SGF (pH 1.2). Satisfactory hydrolysis was observed in SIF (pH 6.8). All prodrugs showed very encouraging hydrolysis rate in 80% human plasma (pH 7.4) and the regeneration of the active drug hydralazine. The synthesized prodrugs showed relative stability in the investigated aqueous solutions and the hydrolysis rates at pH 7.4 are slightly accelerated than those observed in SGF of pH 1.2 and SIF of pH 6.8.

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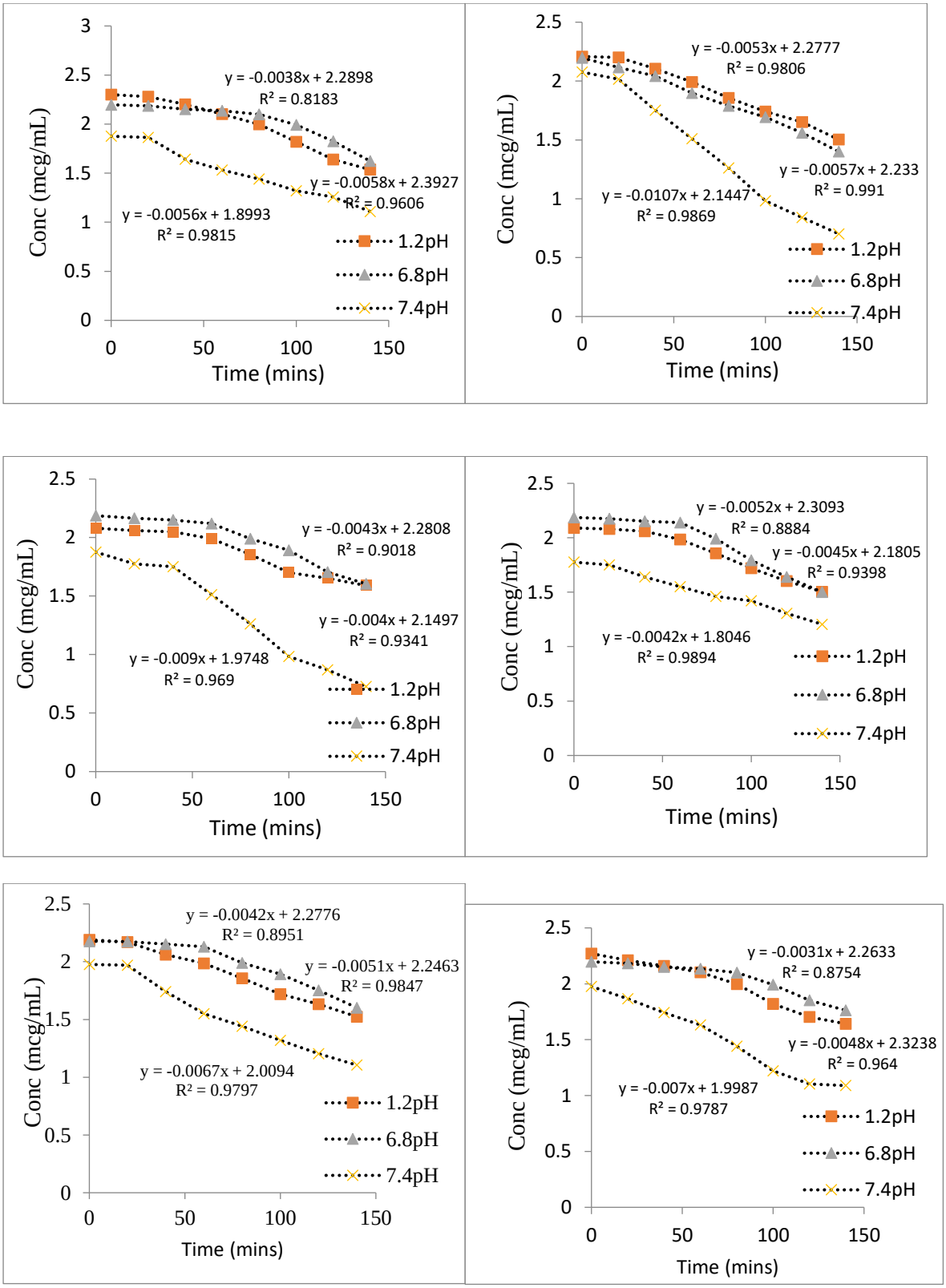

Fig 1: The hydrolysis rate of prodrugs (3a-f) at pH 1.2, 6.8 and 7.4.

Pharmacological evaluation

All the compounds were screened *in vivo* for their antihypertensive activity by DOCA salt-induced model in rat. All the compounds are having remarkable antihypertensive activity, however with a degree of variation. Blood pressure changes in treatment of hypertensive rats with synthesized prodrugs were presented

in table 1. A significant increase in blood pressure was observed in hypertensive rats. All the compounds **3a–f** had shown significant reduction in blood pressure as compared to control hypertensive rats. Among all the derivatives, compound **3e** was found to be more prominent antihypertensive agent.

Table 1: Effect of derivatives on blood pressure by salt loaded induced hypertension

Groups	Average systolic blood pressure (mm Hg) at time (min)					
	0	**15**	**30**	**60**	**120**	**180**
Normal	123 ± 0.57	121 ± 0.60	121 ± 0.76	122 ± 0.60	120 ± 0.55	121 ± 0.70
Hypertension Control	183 ± 0.73	181 ± 0.42	182 ± 0.47	182 ± 0.49	182 ± 0.60	180 ± 0.66
Hydralazine	181 ± 0.66	179 ± 0.55^{ns}	169 ± 1.07***	154 ± 0.33***	136 ± 0.67***	125 ± 0.55***
3a (10 mg/kg,p.o)	181 ± 0.42	179 ± 0.44^{ns}	169 ± 0.49***	152 ± 0.73***	142 ± 0.36***	132 ± 0.42***
3b	182 ± 0.47	182 ± 0.49^{ns}	177 ± 0.57***	164 ± 0.57***	151 ± 0.55***	142 ± 0.42***
3c	181 ± 0.84	180 ± 0.47^{ns}	179 ± 0.47**	163 ± 0.47***	153 ± 0.47***	142 ± 0.60***
3d	182 ± 0.49	181 ± 0.30^{ns}	178 ± 0.47^{ns}	176 ± 0.47***	174 ± 0.30***	173 ± 0.60***
3e	182 ± 0.56	180 ± 0.25^{ns}	178 ± 0.30***	143 ± 0.42***	132 ± 0.83***	130 ± 0.42***
3f	183 ± 0.47	180 ± 0.57	177 ± 0.30^{ns}	148 ± 0.68***	133 ± 0.40***	133 ± 0.72***

Data represented mean ± SEM, (n = 6); data were analyzed relative to control and by one-way ANOVA followed by Dunnett's multiple comparison test $P < 0.05$ (*), $P < 0.01$ (**), $P< 0.0001$ (***)

CONCLUSION

The synthesis of prodrugs of hydralazine was successfully affected in a rather simple and scalable scheme that consists of two steps. The chemical structures of the prodrug and the intermediate were confirmed by FT-IR, ^{1}H NMR, and MS analysis. Absorption bands obtained in IR and NMR spectrum confirmed the formation of imine linkage with hydralazine. Preliminary kinetic study for compounds **3a-f** revealed that compounds are chemically stable to a great extent at pH 1.2 and pH 6.8. While they show chemical hydrolysis at pH 7.4. On the basis of chemical hydrolysis studies, it suggests that prodrugs are showing the slower hydrolysis at the pH 7.4 and having the longer duration of action.

These results indicate that the novel hydralazine prodrug can significantly enhance the oral absorption and bioavailability of hydralazine. Moreover, these novel prodrugs of hydralazine may improve the clinical usefulness, daily doses could be reduce the first pass metabolism.

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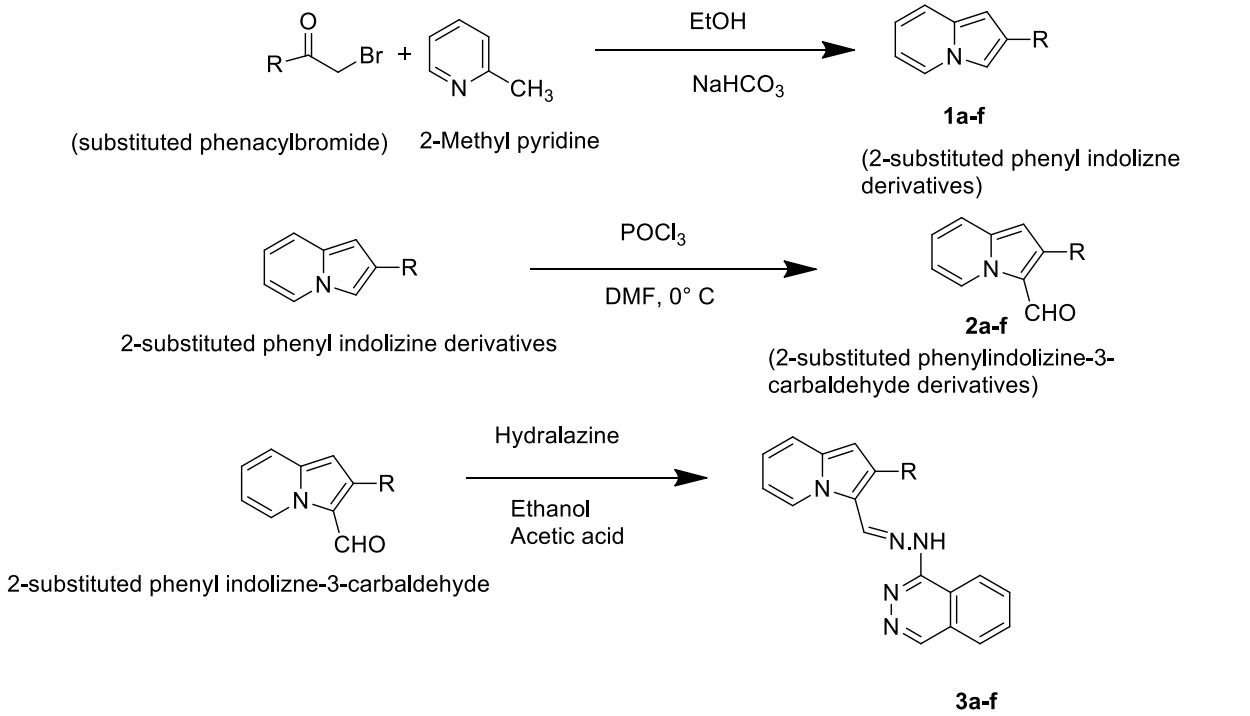

Fig 2: Scheme: Synthesis of Indolizine derivatives

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