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Novel Benzamide Derivatives as Acid Pump Antagonists (APAs)

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ABSTRACT

A series of benzamide derivatives as acid pump antagonists (APAs) was synthesized and the inhibitory activities against H⁺/K⁺ ATPase isolated from pig gastric mucosa were determined. After elaborating on substituents at C₂, C₃, C₄ and C₆ position of the benzamide scaffold, we have observed that the compound 11 is a potent APA with H⁺/K⁺ ATPase, -log(1/IC₅₀) = 1.07 μM.

KEY WORDS: H⁺/K⁺ ATPase, benzamide, acid pump antagonists (APAs)

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INTRODUCTION:

The method of treating peptic ulcer disease has changed dramatically during the last 25 years. Numerous compounds having different modes of action have been used to treat the upper gastrointestinal ulcers. Healing of ulcers may be achieved by three different modes. The first involves the stimulation and regeneration of cells surrounding the ulcer base. Secondly, the ulcer can be protected from gastric acid and pepsin by coating agent. Thirdly the acidity of the gastric juice can be reduced by inhibitors of the gastric acid secretion or by antacids, which neutralize the gastric acidity and by the prostaglandins which inhibit not only gastric acid secretion but additionally stimulate endogenous gastric bicarbonate secretion.^[1] The most successful method to treat the upper gastrointestinal ulcers has been the inhibition of gastric acid secretion. During the last twenty years numerous clinical trials and clinical experiences have demonstrated that inhibition of gastric acid secretion is superior to all other possible methods in promoting ulcer healing and relieving ulcer symptoms. The era of gastric acid inhibitors started experimentally in the early 1970s with the histamine H₂-receptor antagonists. This was followed by gastric proton pump inhibitors (PPIs) about five years later. However, PPIs still continue to have their drawbacks. The currently available PPIs require around 3–5 days to achieve maximum acid inhibition at existent therapeutic doses, primarily due to their chemical structures and irreversible inhibition of H⁺/K⁺-ATPase.^[2,3] Failure to demonstrate a sustained acid inhibition throughout the day and night, in spite of twice daily administration, and nocturnal acid breakthrough (NAB) are found to be common in patients taking PPIs.^[4-6] Therefore, many novel strategies to address the unmet needs of PPI therapy have been investigated, and acid pump antagonists (APAs) could play a promising role, as they provide faster onset and longer duration of action than irreversible PPIs by virtue of their ability to reversibly bind to the proton pump.^[7]

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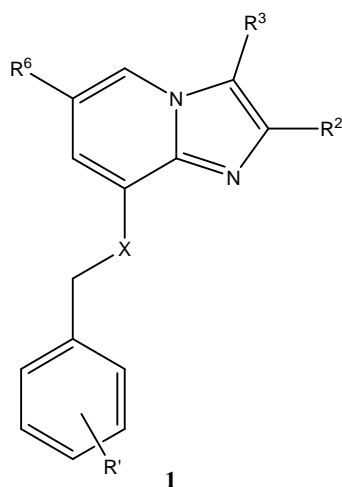
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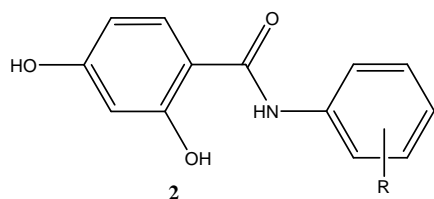
APAs are weak bases and lipophilic that have diverse structures such as, imidazopyridines, pyrimidines, imidazonaphthyridines, quinolines, etc.⁽⁷⁾ The APAs studied most extensively so far rely on substituted imidazo[1,2-a]pyridine derivatives (1) (Fig. 1). And they were shown to inhibit the gastric acid secretion by reversible and K^+ competitive binding to H^+/K^+ ATPase, and they also displayed excellent antisecretory properties.^[8-19]



R^2 = substituted small alkyl, etc.
 R^3 = substituted small alkyl, etc.
 R^6 = H, amide, etc.
 R' = alkyl, halogen, etc.
 X = O, N, etc.

Figure 1. Structure of imidazo[1,2-a]pyridine APAs 1

In the course of our efforts to develop novel and potent APAs, we were able to identify APAs that have a novel scaffold different from the well-known imidazo[1,2-a]pyridine. Here, we report the synthesis and pharmacological evaluation of 2,4-dihydroxy-N-(substituted)phenylbenzamide derivatives of the general formula 2 as APAs (Fig. 2).

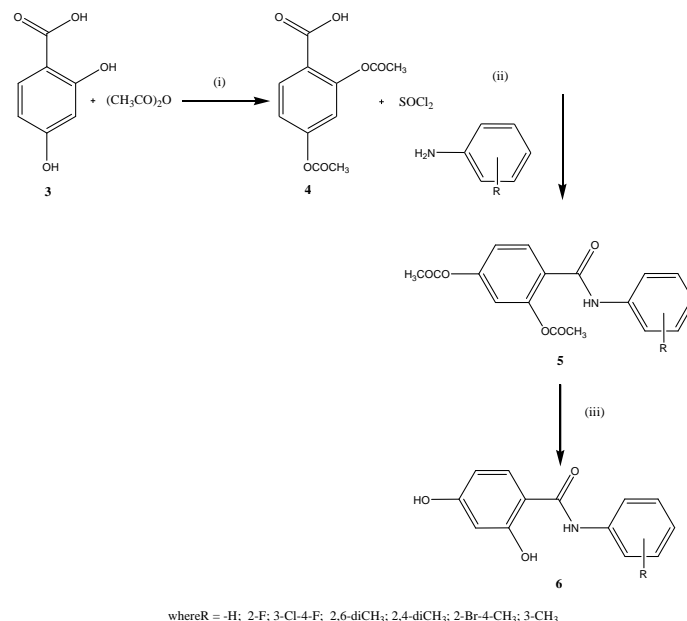


R = -H; 2-F; 3-Cl-4-F; 2,6-diCH₃; 2,4-diCH₃; 2-Br-4-CH₃; 3-CH₃

Figure 2. Structure of 2,4-dihydroxy-N-(substituted)phenylbenzamide APAs 2

In order to synthesize the target compounds 2, the known 2,4-dihydroxy benzoic acid 3, was reacted with acetic anhydride in presence of anhydrous $ZnCl_2$ as a catalyst to provide compound 4. Further, chlorination of compound 4 and

subsequent careful treatment of intermediate acid chloride with different amines led to the benzamide derivatives 5. Finally, hydrolysis of the ester linkage of benzamide derivatives 5 by 0.5 M KOH solution yielded the target compound 2,4-dihydroxy-N-(substituted)phenylbenzamide 2 (Scheme 1).



Scheme 1. Reagents and condition = (i) Anhydrous $ZnCl_2$, Heat 3 hr; (ii) Benzene, Reflux 3 hr; (iii) 0.5 M KOH solution, Reflux 40 min

2. MATERIAL AND METHODS

2.1. General methods

All chemicals and solvents were commercially available and used without further purification. The reactions were monitored by thin-layer chromatography (TLC) using silica plates (TLC Silica gel 60 F₂₅₄, Merck KGaA) and spots were visualized in UV-light at $\lambda = 254$ nm and 366 nm. Melting points (mp) were determined on a VMP-D (Veego make) melting point apparatus and are uncorrected. Mass spectra were obtained on Perkin-Elmer LC-MS PE Sciex API/65. ¹H NMR spectra were recorded on a BRUKER Advance-II 400 MHz spectrometer with TMS as an internal standard.

1.1. 2,4-diacetoxybenzoic acid (4)^[20]

2,4-dihydroxy benzoic acid (3) (5 g, 0.0324 mol) was dissolved in (15.35 mL, 0.162 mol) of acetic anhydride in 100 mL of round bottom flask. To this reaction mixture 0.5 g of anhydrous zinc chloride was added as a catalyst. Heat the reaction mixture on water bath for 3 hours and simultaneously the reaction progress was monitored by TLC. The reaction mixture was kept aside for overnight to get white crystalline product. Filtered the product and the remaining reaction mixture was poured in ice-water. The product formed

was separated by filtration, washed with water and dried. Product was purified by recrystallization from benzene and dichloromethane. (Yield 68%)

2.1.2. General preparation of 2,4-diacetyloxy-N-(substituted)phenylbenzamide (5)^[21]

A mixture of 2,4-diacetyloxy benzoic acid (**4**) (3 g, 0.0126 mol) and thionyl chloride (1.34 mL, 0.0189 mol) was refluxed in 10 mL of anhydrous benzene on water bath for 3 hr. Distilled out the benzene and repeat the same 2-3 times for distilling extra thionyl chloride. To the residue 10 mL of anhydrous benzene and aromatic amines (0.0151 mol) was added and the reaction mixture was refluxed for 4-5 hours. Distilled out the benzene and the residue was treated with dilute HCl to remove extra aniline. The solid was filtered, washed with water and dried.

2.1.3. General preparation of 2,4-dihydroxy-N-(substituted)phenylbenzamide (6)^[22]

2,4-diacetyloxy-N-(substituted)phenylbenzamide (**5**) (1 g, 0.0032 mol) was added to 40 mL of 0.5 mol aqueous potassium hydroxide solution and then refluxed for 40 min. Cooled to room temperature and 1 N aqueous HCl solution was added to get pH 4 and the resultant ppts was filtered. The product was washed with water and dried. Product was purified by recrystallization from toluene or chlorobenzene or mixture of methanol and water.

2,4-Dihydroxy-N-phenylbenzamide (7). IR (KBr, cm^{-1}): 3330.84 (O-H stretching of phenol), 3321.19 (N-H stretching of amide), 3062.75 (C-H stretching), 1631.67 (CO stretching of amide), 1566.09 (N-H stretching of amide); Mass (Methanol) (m/z): 230.0 (M+1); ^1H NMR (400 MHz, DMSO): δ 6.32-6.36 (d, 2 H, ArH), 7.06-7.10 (m, 1 H, ArH), 7.28-7.32 (m, 2 H, ArH), 7.64-7.66 (m, 2 H, ArH), 7.89 (d, 1 H, ArH), 9.99 (s, 1 H, OH, D₂O exchange), 10.05 (s, 1 H, OH, D₂O exchange), 12.28 (s, 1 H, NH, D₂O exchange)

2,4-Dihydroxy-N-(2-fluorophenyl)benzamide (8). IR (KBr, cm^{-1}): 3544.92 (O-H stretching of phenol), 3369.41 (N-H stretching of amide), 3242.12 (C-H stretching), 1731.96 (CO stretching of amide), 1610.45 (N-H stretching of amide); Mass (Methanol) (m/z): 248.2 (M+1), 249.2 (M+2); ^1H NMR (400 MHz, DMSO): δ 6.39 (d, 1 H, ArH), 6.41-6.42 (d, 1 H, ArH), 7.14-7.23 (m, 2 H, ArH), 7.28-7.33 (m, 1 H, ArH), 7.86-7.88 (m, 1 H, ArH), 8.13-8.17 (m, 1 H, ArH), 10.22 (bs, 1 H, OH, D₂O exchange), 10.47 (s, 1 H, OH, D₂O exchange), 11.98 (s, 1 H, NH, D₂O exchange)

2,4-dihydroxy-N-(3-chloro-4-fluorophenyl)benzamide (9). IR (KBr, cm^{-1}): 3438.84 (O-H stretching of phenol), 3352.02 (N-H

stretching of amide), 3120.61 (C-H stretching), 1593.09 (CO stretching of amide), 1556.45 (N-H stretching of amide); Mass (Methanol) (m/z): 282.1 (M+1), 285.2 (M+4); ^1H NMR (400 MHz, DMSO): δ 6.32-6.33 (d, 2 H, ArH), 7.18-7.22 (t, 1 H, ArH), 7.55-7.59 (m, 1 H, ArH), 7.82-7.84 (d, 1 H, ArH), 7.97-7.99 (q, 1 H, ArH), 10.53 (s, 1 H, OH, D₂O exchange)

2,4-dihydroxy-N-(2,6-dimethylphenyl)benzamide (10). IR (KBr, cm^{-1}): 3353.98 (O-H stretching of phenol), 3236.33 (N-H stretching of amide), 2977.89 (C-H stretching), 1606.59 (CO stretching of amide), 1535.23 (N-H stretching of amide); Mass (Methanol) (m/z): 258.2 (M+1).

2,4-dihydroxy-N-(2,4-dimethylphenyl)benzamide (11). IR (KBr, cm^{-1}): 3577.71 (O-H stretching of phenol), 3382.91 (N-H stretching of amide), 3085.89 (C-H stretching), 1610.65 (CO stretching of amide), 1595.02 (N-H stretching of amide); Mass (Methanol) (m/z): 258.0 (M+1); ^1H NMR (400 MHz, DMSO): δ 2.19 (s, 3 H, CH₃), 2.27 (s, 3 H, CH₃), 6.35-6.38 (d, 1 H, ArH), 6.33 (d, 1 H, ArH), 7.00-7.02 (d, 1 H, ArH), 7.07 (s, 1 H, ArH), 7.49-7.51 (d, 1 H, ArH), 7.86-7.88 (d, 1 H, ArH), 9.97 (s, 1 H, OH, D₂O exchange), 10.14 (s, 1 H, OH, D₂O exchange), 12.35 (s, 1 H, NH, D₂O exchange)

2,4-dihydroxy-N-(2-bromo-4-methylphenyl)benzamide (12). IR (KBr, cm^{-1}): 3379.05 (O-H stretching of phenol), 3292.26 (N-H stretching of amide), 3029.96 (C-H stretching), 1593.09 (CO stretching of amide), 1517.87 (N-H stretching of amide); Mass (Methanol) (m/z): 322.1 (M+1), 324.1 (M+2); ^1H NMR (400 MHz, DMSO): δ 2.29 (s, 3 H, CH₃), 6.38-6.41 (m, 2 H, ArH), 7.19-7.22 (d, 1 H, ArH), 7.52 (s, 1 H, ArH), 7.86-7.88 (s, 1 H, ArH), 8.06-8.08 (d, 1 H, ArH), 10.18 (s, 1 H, OH, D₂O exchange), 10.44 (s, 1 H, OH, D₂O exchange), 11.95 (s, 1 H, NH, D₂O exchange)

2,4-dihydroxy-N-(3-methylphenyl)benzamide (13). IR (KBr, cm^{-1}): 3353.98 (O-H stretching of phenol), 3301.91 (N-H stretching of amide), 3051.18 (C-H stretching), 1602.74 (CO stretching of amide), 1550.09 (N-H stretching of amide); Mass (Methanol) (m/z): 244.1 (M+1); ^1H NMR (400 MHz, DMSO): δ 2.31 (s, 3 H, CH₃), 6.32 (d, 1 H, ArH), 6.36-6.39 (d, 1 H, ArH), 6.93-6.95 (d, 1 H, ArH), 7.21-7.25 (t, 1 H, ArH), 7.44-7.46 (d, 1 H, ArH), 7.50 (s, 1 H, ArH), 7.88-7.90 (d, 1 H, ArH), 10.08 (s, 1 H, OH, D₂O exchange), 10.20 (s, 1 H, OH, D₂O exchange), 12.29 (s, 1 H, NH, D₂O exchange)

2.2. Biological Evaluation

2.2.1. Preparation of pig gastric H⁺,K⁺-ATPase^[23,24]

Porcine stomachs were obtained from Ahmedabad Municipal Corporation, Jamalpura. The fundus of each stomach was

isolated, rinsed with tap water, and washed with 3M NaCl to remove superficial cells, cell debris, and mucus. Then the oxyntic cell-rich mucosa was scraped off and homogenized in a homogenizing buffer 0.25 M sucrose, 5 mM PIPES/Tris, pH 6.8, 1 mM EDTA, and 1 mM EGTA. The homogenate was centrifuged at 20,000 *g* for 30 min, and the supernatant was further centrifuged at 1,00,000 *g* for 90 min. The pellet was resuspended in homogenizing buffer, after which it was layered over 7.5% (w/w) Ficoll in the homogenizing buffer and centrifuged at 1,00,000 *g* for 60 min. The microsomal fraction (concentrated in the middle layer) was collected, diluted with homogenizing buffer, and centrifuged again at 1,00,000 *g* for 90 min. Then the pellet was collected and suspended in homogenizing buffer at a final protein concentration of 0.5 mg/mL, after which the suspension was stored at -80°C until its protein content was determined with a Lowry protein assay method.

2.2.2. Measurement of H^+/K^+ -ATPase activity²³

Gastric H^+/K^+ -ATPase activity was measured by quantifying the release of inorganic phosphate from ATP^{25} in a 96 well format. Reactions were performed in a reaction mixture of 50 μL containing 2.5 mg/L vesicles, 50 mM Hepes-Tris (pH 7.4), 5 mM MgCl_2 , 8 mM KCl, 10 μM valinomycin in the presence of the test compound or vehicle. After preincubation at 37°C for 30 min, the reaction was initiated by the addition of ATP at a final concentration of 0.2 mM and incubation at 37°C for 20 min. The reaction was stopped by adding 15 μL of dye reagent containing 0.1% w/v malachite green, 1.5% w/v hexaammonium molybdate, and 0.2% v/v Tween 20 in 4 N H_2SO_4 , after which the absorbance was measured at 620 nm with a ELISA reader. K^+ -dependent ATPase activity was calculated as the difference between the activity in the presence and absence of KCl. For the controls with 0% inhibition and 100% inhibition, enzymatic reactions were carried out in the presence of 1% DMSO and omeprazole (0.56-14 μM), respectively.

3. RESULTS AND DISCUSSIONS

All 2,4-dihydroxy-N-(substituted)phenylbenzamide 7-14 as well as omeprazole were evaluated in a assay against H^+/K^+ -ATPase from pig gastric mucosa. The results are summarized in Table 1. The unsubstituted benzamide (7) was not active compared to the reference compound omeprazole. On the other hand, the *in vitro* activity of compound 7 was shown, when one or two alkyl substituents were introduced on the phenyl ring of benzamide. Activity was mainly dependent on the position of the substituent. When both electron donating and electron withdrawing substituent were introduced on

compound (7), the activity of compound (12) decreases compared to compounds containing only electron withdrawing or electron donating group (8, 10, 11, 13).

Table 1. Inhibition of H^+/K^+ -ATPase by compounds 7-13

Compound	R	H^+/K^+ -ATPase assay -log(1/IC ₅₀)
Omeprazole		
7	H-	NA
8	2-F-	1.27
9	3-Cl-4-F-	NA
10	2,6-diCH ₃ -	1.29
11	2,4-diCH ₃ -	1.07
12	2-Br-4-CH ₃ -	1.97
13	3-CH ₃ -	1.29

NA = not active

4. CONCLUSION

In summary, we have reported a general route for the preparation of 2,4-dihydroxy-N-(substituted)phenylbenzamide. One compound of this series (11) was shown to have a superior *in vitro* activity as antagonists of the gastric H^+/K^+ -ATPase compared to the known anti ulcer agent omeprazole.

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