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Discovery of a dihydropyrimidine series of molecules that selectively mimic the biological actions of calcitonin

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Abstract—The use of a multiplex mimetic assay led us to identify 1,4-dihydropyrimidines with potent and selective calcitonin receptor mimetic activity. Subsequent modification of the dihydropyrimidine scaffold led to a series of molecules that were efficacious in a neonatal mouse calvaria in vitro model. Dihydropyrimidine **5h**, in particular, was identified as a calcitonin mimetic (EC_{50} = 6 μM), active in-vivo in the Weanling rat model when administered subcutaneously.

1. Introduction

Calcitonin, a 32 amino acid polypeptide hormone secreted by the thyroid and thymus glands, plays an important role in inhibiting bone resorption through the mediation of osteoclasts. By inhibiting bone resorption and promoting renal calcium excretion, calcitonin has therapeutic applications in a variety of clinical disorders, including hypercalcemia associated with Paget's disease[1] and osteoporosis.[2,3] Although, salmon-calcitonin (s-CT) has been effective in decreasing bone resorption via parenteral administration, its widespread use is limited because of a short half-life and lack of compliance due to the need for multiple injections. To date, only the Suntory group[4,5] and the Zymogenetics group[6] have reported non-peptidic calcitonin mimetics. Therefore, we sought to overcome the problems associated with s-CT by identifying novel non-peptide mimetics of calcitonin that are selective and orally bioavailable.

To screen our internal library, a multiplex mimetic cell based assay[7] was designed for high-throughput screening. In an effort to differentiate activity amongst similar G-protein coupled receptors, 6 cell lines—calcitonin receptor-2 (CTR-2; clone #33), glucogen-like peptide 1 (GLP1-7; clone #7), gastric inhibitory polypeptide (GIP-1; clone #1), parathyroid hormone receptor 1 (EPTH-1-1; clone #1) and calcitonin gene related peptide-1 (CGRP1-7; cone 7)—were cloned onto the human embryonic kidney (HEK 293) cell line and plated together in one assay well.

The compounds (**1**–**4**) in Figure 1 are exemplars of an active series of 1,4-dihydropyrimidines that stimulated

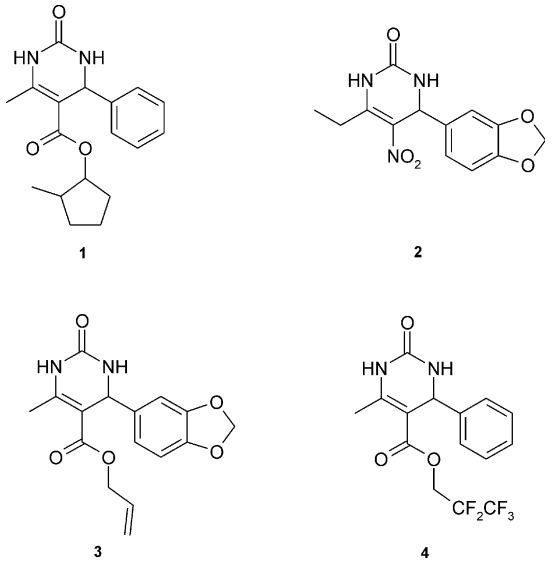

Figure 1. Structures of 1,4-dihyropyrimides (**1**–**4**) from the multiplex mimetic assay.

Keywords: Calcitonin; Dihydropyrimidine; GPCR.
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Table 1. Fold increase[a] data for compounds **1–4** in the multiplex cell-based assay[7]

Compd	Muliplex[b] (6 cell lines)	CTR-2HEK	GLP1-7HEK	GIP-1HEK	GLU-1HEK	EPTH1-1HEK	CGRP1-7HEK
1	4.5/5.7	8	NA	NA	NA	NA	NA
2	4.7/4.0	12	NA	NA	NA	NA	NA
3	4.7/3.2	8	NA	NA	NA	NA	NA
4	5.1/4.4	9	NA	NA	NA	NA	NA

[a] pmol of cAMP accumulation simulated by the compound/basal level of cAMP produced by the cell line @ 25 μM.
[b] Data for multiple screens; NA = no activity.

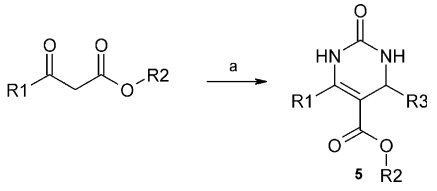
cAMP accumulation in HEK 293 cells expressing the CTR-2 ligand. The activity and selectivity of these compounds are outlined in Table 1 with activity shown as a measurement of fold-induction (FI).

In a single effort, we were able to identify a lead series that demonstrated selectivity against the parental cells and additional GPCR's.

Herein we report the synthesis and biological activity of a 1,4-dihydropyrimides series. The chemistry necessary to develop structure–activity relationships was accomplished using the well-documented Biginelli reaction, shown in Schemes 1 and 2.

2. Chemistry

1,4-dihydropyrimidines (**5**) of interest were assembled via a multi-component reaction of an aldehyde, urea, and β-keto ester as shown in Scheme 1.[8,9] Using parallel synthesis, we utilized this reaction for the rapid synthesis of diverse compounds. In the instance when R_2 = benzyl (**5c**), catalytic hydrogenation afforded carboxylic acid (**6**). Standard coupling conditions of (**6**) with benzyl alcohols (**7**) provide target compounds (**8**), as shown in Scheme 2.

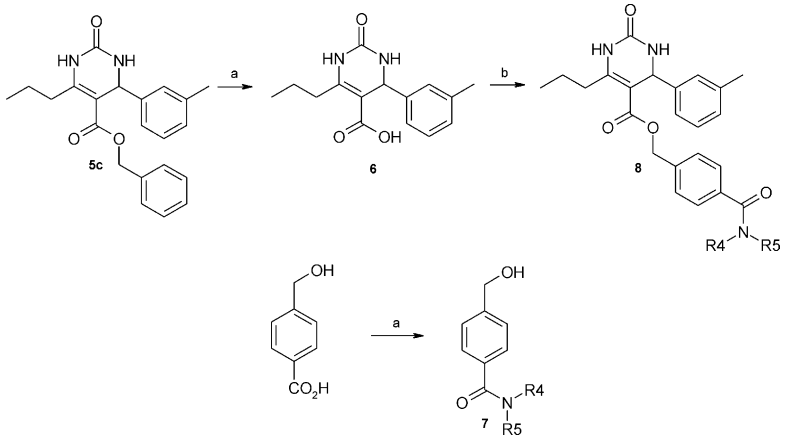


Scheme 1. (a) ArCHO, urea, MeOH, cat. HCl, 65 °C.

3. Results and discussion

To elucidate the structure–activity relationship of the dihydropyrimidine series, modifications of the (R_1) C-6 side-chain, the ester appendage (R_2) and the C-4 aryl (R_3) where examined. The cAMP accumulation in HEK 293 cells in the CTR-2 receptor was measured and recorded as fold increase.

Compound **5a**, shown in Table 2, was our first entry into the 1,4-dihydropyrimidine series and by simply modifying the ester linkage and the C-4 aryl moiety (R_3), we achieved a 4 to 5-fold improvement over compounds **1–4**, in regards to fold-increase. Addition of a 3-methyl phenyl (**5b**) onto R_3 afforded a compound with a modest increase in potency. Lengthening the R_1 alkyl chain from a methyl (**5b**) to a propyl (**5c**) resulted in a 2 to 3-fold increase in activity, however, further increases led to compounds which were poorly soluble in the biological media, exemplified by compound **5d**. The benzyl ester and the ethyl ester (**5e**) analogues were equipotent. Replacement of the 3-methyl phenyl moiety (**5e**) with a 3-chloro-phenyl group (**5f**) or the 3,5-dimethyl phenyl group (**5g**) at R_3 did not significantly alter activity. Compounds with hydrogen bonding groups, such as a 4-methoxy benzyl (**5h**) at R_2 were synthesized and equipotent to compound **5c**, and further manipulations led to the 4-methylester benzyl analogue **5i**, the



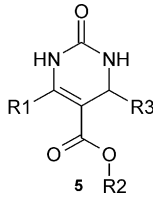
Scheme 2. (a) H_2, Pd/C, EtOAc:EtOH (9:1), 25 °C; (b) CH_2Cl_2:DMF (2:1), EDC, cat. DMAP, **7**, 65 °C; (c) CH_2Cl_2, EDC, R_4R_5NH, 25 °C.

first low micromolar compound in the series. Replacement of the ester in **5i** with an acid moiety (**5j**) did not significantly change the activity.

Several compounds were evaluated in a bone organ culture assay. This assay is an in vitro bone resorption assay in which the % inhibition of parathyroid hormone (PTH) stimulated bone resorption is measured in neonatal mouse calvaria.[10] Figure 2 demonstrates the efficacy of compounds **5a**, **b**, **e**, and **h** at 10, 30 and 100 μM, as well as s-CT as a control at 0.01, 1 and 3 nM. Though the dihydropyrimidines are several orders of magnitude less potent than s-CT, there is a clear dose-dependent response toward the inhibition of bone resorption in the calcium-45 labeled mouse calvaria.

Compound **5h** was further evaluated in a Weanling rat study.[11] Weanling rats are a species that undergo rapid bone remodeling, thus have high rates of bone resorption. The rats are placed on a low calcium diet prior to the study to ensure that the source of calcium in the blood will be dependent on bone resorption and not the diet. After dosing the compounds, the urine is collected and analyzed for urinary deoxypyridinolines, a common bone marker. Figure 3 depicts s-CT, dosed at 1 mpk, and compound **5h**, dosed at 100 mpk, both subcutaneously.

Table 2. The fold increase and EC_{50} of 1,4-dihydropyrimidine **5**



5

Compd	R_1	R_2	R_3	FI[a]	EC_{50} (μM)[a]
5a	CH_3	$CH_2C_6H_5$	Ph	17	>20 ($n=1$)
5b	CH_3	$CH_2C_6H_5$	3-CH_3-Ph	23	15 ($n=1$)
5c	$(CH_2)_2CH_3$	$CH_2C_6H_5$	3-CH_3-Ph	36	5.2 ($n=1$)
5d	$(CH_2)_4CH_3$	CH_2CH_3	3-CH_3-Ph	31	ND[b]
5e	$(CH_2)_2CH_3$	CH_2CH_3	3-CH_3-Ph	35	7.5 ($n=2$)
5f	$(CH_2)_2CH_3$	CH_2CH_3	3-Cl-Ph	21	6.8 ($n=1$)
5g	$(CH_2)_2CH_3$	CH_2CH_3	3,5-CH_3-Ph	34	9.5 ($n=1$)
5h	$(CH_3)_2CH$	$CH_2C_6H_4$-4-OCH_3	3-CH_3-Ph	33	5.8 ($n=4$)
5i	$(CH_2)_2CH_3$	$CH_2C_6H_4$-4-CO_2CH_3	3-CH_3-Ph	42	1.0 ($n=2$)
5j	$(CH_2)_2CH_3$	$CH_2C_6H_4$-4-COOH	3-CH_3-Ph	61	3.4 ($n=7$)
sCalcitonin				40	0.003

[a] Activity of compounds (assessed at 75 μM) were expressed as pmol of cAMP and fold increase above vehicle ±5%. Potency of the compounds were determined by performing a dose–response curve starting with a concentration of 75 μM with 1:2 dilution (8 to 16 points curve) and reported result as 50% of EMAX (EC_{50}), ±5%.
[b] ND = not determined due to lack of solubility.

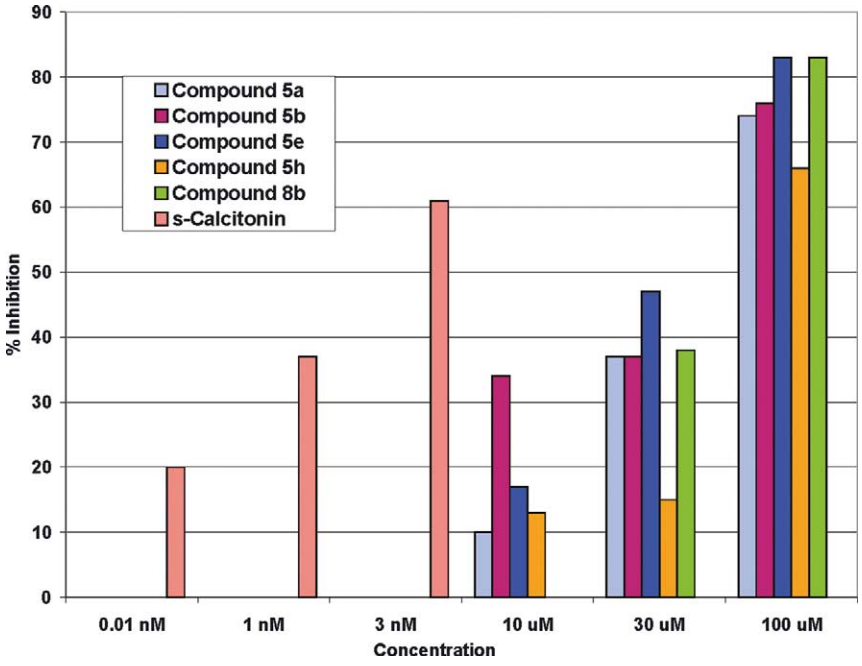


Figure 2. % Inhibition of PTH stimulated response for s-CT, **5a**, **5b**, **5e**, **5h** and **8b**.

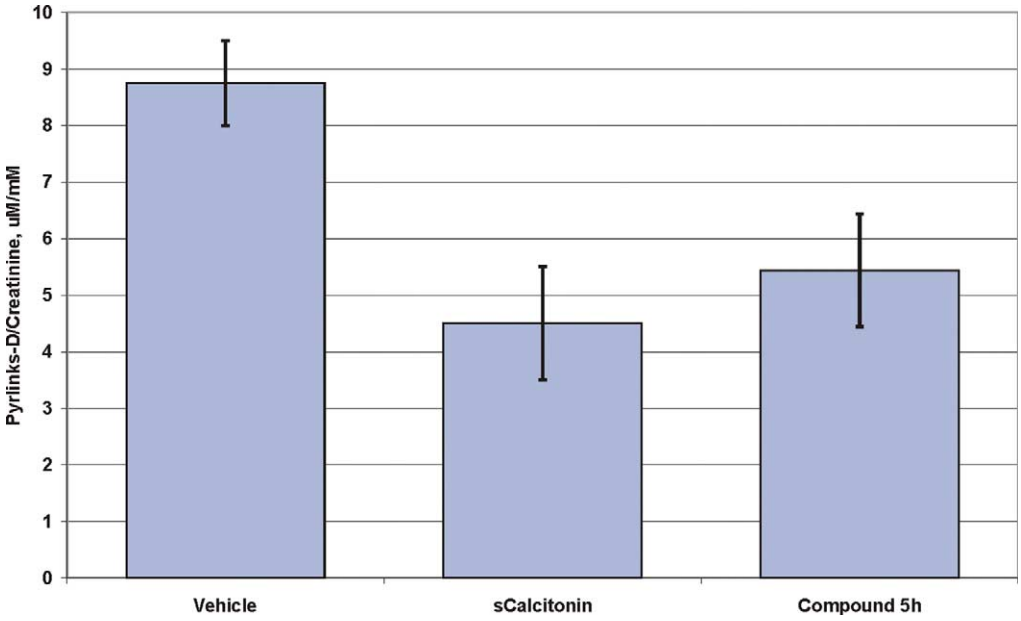


Figure 3. Urinary deoxypyridinolines collected from Weanling rat for s-CT and compound **5h**.

Though the dosage of s-CT and compound **5h** are significantly different, we were able to achieve efficacy similar to s-CT in the Weanling rat, indicative that the dihydropyrimidine series had potential as a calcitonin mimetic.

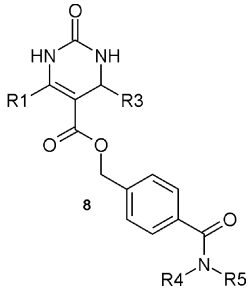
The low solubility of the dihydropyrimide series, exemplified by compound **5i** (pH 2; 25 μg/mL; pH 7.4; 20 μg/mL), and low oral bioavailability in rats, exemplified by compound **5e** (2%, dosed at 100 mpk, po and 2 mpk, iv), led us to seek to improve the physiochemical properties of the series.

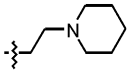
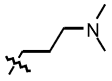
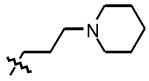
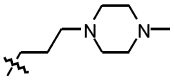
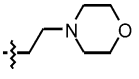
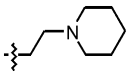
Based on the SAR generated, the most amenable point for modification in the molecule was at R_2—the benzyl ester. In an effort to address both the solubility and potency issues, several compounds were prepared. As shown in Table 3, attachments of an ethyl piperidine (**8b**), dimethyl-aminopropyl (**8c**), or propyl piperidine (**8d**) moieties afforded compounds equipotent to compound **5i**. As desired, compounds in Table 3 also showed improved solubility, with 184 μg/mL (pH 2) and 274 μg/mL (pH 7.4); and 500 μg/mL (pH 2) and 270 μg/mL (pH 7.4) for compounds **8b** and **8c**, respectively. Compound **8b** was evaluated for oral bioavailability, and showed marked improvement as well, with 65% (30 mpk po, 1 mpk, iv) and 13% (10 mpk, po, 1 mpk, iv) in rats and rhesus monkeys, respectively.

Compound **8b** was evaluated for its ability to inhibit PTH stimulated bone resorption in the mouse calvaria model, as shown in Figure 2. As shown, **8b** inhibits bone resorption in a dose-dependent manner at concentrations of 30 and 100 μM.

Compound **8b** was next evaluated orally in the Weanling rat model. Unfortunately, when **8b** was administered po at 100 mpk, there was no significant decrease

Table 3. The fold increase and EC_{50} of dihydropyrimidine series **8**



Compd	R_4	R_5	FI[a]	EC_{50} (μM)[a]
8a	CH_3	CH_3	40	3.4 ($n=1$)
8b	H		51	1.3 ($n=5$)
8c	H		55	1.4 ($n=2$)
8d	H		52	1.9 ($n=2$)
8e	H		55	4.3 ($n=1$)
8f	H		50	4.3 ($n=1$)
8g	CH_3		55	4.4 ($n=1$)
SCalcitonin			40	0.003

[a] Activity of compounds (assessed at 75 μM) were expressed as pmol of cAMP and fold increase above vehicle ±5%. Potency of the compounds were determined by performing a dose–response curve starting with a concentration of 75 μM with 1:2 dilution (8 to 16 points curve) and reported result as 50% of EMAX (EC_{50}), ±5%.

in the plasma calcium and serum pyridinoline bone markers.[12]

4. Conclusion

We have identified a novel series of calcitonin mimetics containing a dihydropyrimidine nucleus. These compounds stimulate cAMP accumulation in HEK 293 cells expressing the CTR-2 receptor and are selective against other G-protein coupled receptors such as GLU-1 and EPTH-1. Several analogues in the series inhibited PTH stimulated bone resorption in a bone organ culture assay in a dose-dependent manner and compound **5h** was efficacious in a Weanling rat model when administered subcutaneously. While we were able to significantly improve the oral bioavailability and potency, while retaining efficacy in the mouse calvaria model, preliminary oral evaluation of **8b** in the Weanling rat study showed no in vivo efficacy. While these compounds did not demonstrate the in vivo efficacy we desired, they may in fact serve as a template for future small molecule calcitonin mimetic ligands to be developed.

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7. A 'multiplex' cell based assay was developed to increase the screening throughput for non-peptide mimetics of several G-protein coupled receptors. Cloned stable HEK293 cell lines mentioned in the text were combined in each assay well and assayed for their ability to stimulate cAMP accumulation in the cell mixture. All compounds were tested in parallel in the HEK293s parental line that did not contain target receptors. If a test compound stimulated cAMP in the multiplex cells and was inactive in HEK293s parental line, the test compound was deemed active and moved to a deconvolution assay. In the deconvolution assay, the active compound was run again in a cAMP accumulation assay using each of the six cloned HEK293 cell lines individually. This 'deconvolution' assay determined activity and selectivity of the test compound.
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12. While compound **8b** is orally bioavailable, the PK analysis (AUC 0.9 um-Hrs; C_{max} 0.5 uM) suggests that, given the modest potency, drug levels required for efficacy may not have been achieved.