Bioorganic & Medicinal Chemistry Letters 22 (2012) 5681–5684

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Orally available pyridinylpyrimidine derivatives as novel RANKL-induced osteoclastogenesis inhibitors

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ARTICLE INFO

Article history:
Received 22 May 2012
Revised 27 June 2012
Accepted 27 June 2012
Available online 6 July 2012

Keywords:
RANKL
Inhibitor
Osteoclastogenesis
Pyrimidine
Benzyl

ABSTRACT

An HTS campaign led to the identification of 4-pyrroldino-2-(pyridin-2-yl)pyrimidine compound **1** as an RANKL-induced osteoclastogenesis inhibitor. The compound **1** showed high clearance values in microsomes, however. Modification of the pyrrolidino group to a benzylamino group improved human microsomal stability with a slight loss of in vitro activity. Substitution at the *ortho* position of the benzyl group ameliorated in vitro activity, and further fluorination of the benzyl group improved microsomal stability in rodents. Representative members of this series, compounds **20** and **23,** exhibited efficacy in RANKL-induced osteopenic mice when administered orally at 0.3 mg/kg.

Osteoclasts are multinucleated cells derived from a monocyte lineage which express tartrate-resistant acid phosphatase (TRAP) on the cell surface as a differentiation marker, and play an essential role in bone resorption.[1] Osteoclasts and the bone forming cells, osteoblasts, cooperate in bone remodeling to maintain the mechanical competence of bone. Osteoblasts supply a critical factor, receptor activator of nuclear factor-κB (RANK) ligand (RANKL), which binds to its cellular acceptor, RANK, and triggers a signal transduction and subsequent gene expression. This in turn results in differentiation of the osteoclast precursor into TRAP-positive, multinucleated bone-resorptive cells.[2] Aging or diseases, including osteoporosis, bone metastasis and rheumatoid arthritis, cause excessive RANKL expression, which leads to abnormally increased bone resorption.[3,4]

Inhibition of osteoclastic activity is a primary therapeutic approach to bone loss.[1b] Bisphosphonates (BPs) are the major anti-resorptive therapy currently available.[5] These agents accumulate almost exclusively in skeletal sites and induce apoptosis in osteoclasts. The efficacy of BPs has been recognized, but gastrointestinal disorders are reported as common adverse effects. In addition, BP-related osteonecrosis of the jaw is a severe side effect which is difficult to treat.[6] Estrogen replacement therapy and selective estrogen receptor modulators (SERMs) also suppress bone resorption,[1b] but these therapies function only in the prevention and treatment of osteoporosis associated with estrogen deficiency in women, and estrogen replacement therapy increases the risk of cancer. These problems with current treatments emphasize the need for novel anti-resorptives.

The RANKL/RANK signaling pathway is a promising target for the development of effective osteoclastogenesis inhibitors. Denosumab, a humanized anti-RANKL antibody, is a new agent for the treatment of osteoporosis and other bone diseases.[4] In addition to biologics, which are administered parenterally, orally available small-molecule inhibitors might also be valuable in the treatment of bone loss, and several groups have in fact recently reported small-molecule inhibitors of RANKL-induced osteoclastogenesis.[7,8] Identification of potent small molecules of practical use has been challenging, however; efficacy through oral administration is not disclosed in most cases, and no clinical trial of small-molecule RANKL-induced osteoclastogenesis inhibitors has been reported.

Here, we report the modification of a high throughput screening (HTS) hit (**1**) which resulted in the discovery of a series of orally bioavailable anti-resorptive agents. An HTS campaign was performed using a RANKL-induced TRAP promoter-dependent reporter gene assay.[8] We successfully identified 4-pyrrolidino-2-(pyridin-2-yl)pyrimidine derivative **1** as an HTS hit with an IC_{50}

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http://dx.doi.org/10.1016/j.bmcl.2012.06.087

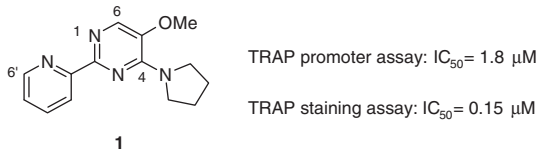


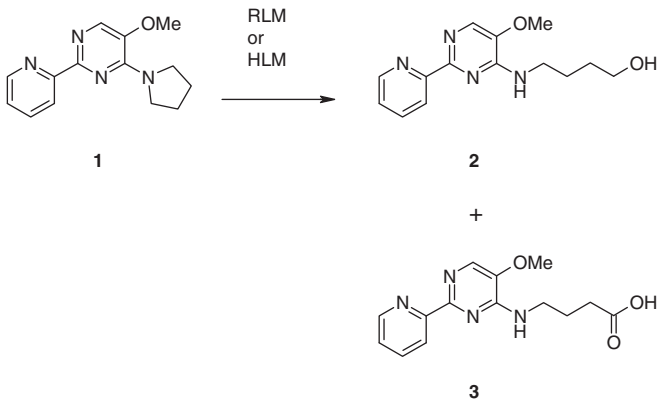
Figure 1. Structure and anti-osteoclastogenic activity of HTS hit **1**.

value of 1.8 μM in the promoter assay (Fig. 1). Inhibitory activity against RANKL-induced osteoclastogenesis was confirmed with an IC_{50} of 0.15 μM in a TRAP staining assay in RAW264 cells, an immortalized mouse macrophage-like cell line possessing the ability to differentiate into osteoclasts.[9] However, compound **1** showed high clearance values in liver microsomes of mouse, rat and human (abbreviated as MLM, RLM and HLM, respectively) in vitro. We therefore conducted a preliminary search for the structure–activity relationships (SAR) of in vitro activity against osteoclastogenesis, and also investigated metabolic pathways in microsomes. The following results were obtained:

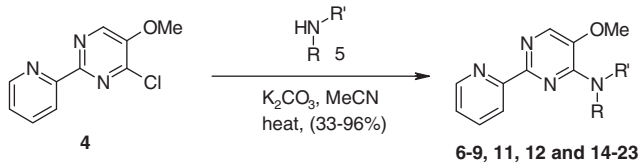
1. The 2-pyridinyl group on the pyrimidine ring was essential for inhibitory activity.
2. Substitution at either the 6-position of the pyrimidine ring or the 6′ position in the 2-pyridinyl group attenuated potency.
3. The pyrrolidine ring of **1** was identified as the main site of metabolism in both RLM or HLM in vitro by LC–MS/MS analysis (Scheme 1). One of the C–N bonds in the pyrrolidine ring was cleaved to generate two oxygenated metabolites **2** and **3**.[10]

On the basis of these preliminary results, we embarked on a modification of the pyrrolidine ring to improve both potency and metabolic stability.

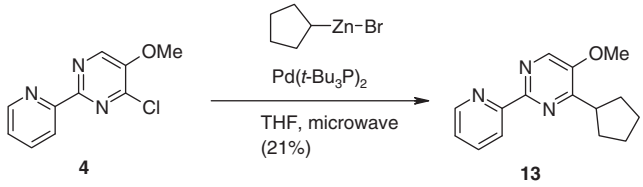
The general synthetic route for various 4-amino derivatives was efficient in only one step, as illustrated in Scheme 2. A commercially available chloropyrimidine **4** was reacted with amines **5** to afford the compounds **6–9, 11, 12** and **14–23** in moderate to high yields. The cyclopentyl compound **13** was prepared by Negishi coupling reaction of the chloropyrimidine **4** with cyclopentylzinc bromide under microwave irradiation as shown in Scheme 3.[11] The synthesized compounds were evaluated in vitro to investigate both their inhibitory activity against RANKL-induced osteoclastogenesis and microsomal stability, as shown in Table 1. The piperidine **6** and *N*-methyl-*N*-cyclopentylamine **9** showed equipotent activity with **1**, although clearance values in liver microsomes were increased. The azabicyclic compound **8** retained activity and stability, while conversion of the pyrrole group into azepane (**7**) decreased activity. These results suggested that replacement of the pyrrolidine



Scheme 1. Major metabolites of compound **1** in both RLM or HLM.

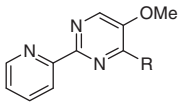


Scheme 2. Synthesis of compounds **6–9**, **11**, **12** and **14–23**.



Scheme 3. Synthesis of compound **13**.

Table 1
Potency in TRAP staining assay and microsomal stability of 4-amino/4-cyclopentyl pyrimidines[a]



Compd	R	TRAP staining[a] IC_{50} (M)	$Cl_{int,\ in\ vitro}$ (ml /min/kg)[b]		
			MLM	RLM	HLM
1	—N	0.15	>1000	987	458
6	—N	0.10	N.T.	>1000	657
7	—N	0.69	N.T.	N.T.	N.T.
8	—N	0.24	N.T.	734	512
9	—N(*c*-Pent)(Me)	0.18	N.T.	>1000	775
10	—N	>2	N.T.	N.T.	N.T.
11	—N	1.0	>1000	210	178
12	—N	1.4	>1000	395	310
13	*c*-Pent	1.8	N.T.	>1000	749
14	—NH—*c*-Pent	0.57	>1000	202	210
15	—NH—Ph	0.42	911	387	67

[a] Inhibitory activity of RANKL-induced osteoclastogenesis using RAW264 cells ($n \geqslant 3$).
[b] Average of two experiments. N.T.: Not tested.

ring with tertiary amino groups possessing simple aliphatic substituents might not improve metabolic stability sufficiently while retaining inhibitory activity. With the aim of blocking metabolism, introduction of electro-negative atoms and depletion of nitrogen were implemented. Disappointingly, embedding an oxygen atom in the ring as morpholine (**10**) resulted in the complete loss of activity. Fluorination of the pyrrolidine ring (**11**, **12**) and replacement of the nitrogen with a carbon (**13**) also reduced inhibitory activity, although the fluorination showed a tendency to improve

microsomal clearance. These observations indicated that the electro-donating ability of the nitrogen atom is an important factor for activity. Next, the potency of the secondary amino groups was investigated. The cyclopentylamino derivative **14** showed decreased activity in comparison to **9**, implying that the presence of two substituents of tertiary amino groups affected the inhibitory activity positively. The benzylamino compound **15** slightly decreased activity, but the microsomal stability of **15** was improved sufficiently in human and moderately in rodents.

Based on these results and the feasibility of benzyl modification, we selected the benzyl derivative **15** as a lead and planned a strategy to improve inhibitory activity. To compensate for the loss of one substituent on the nitrogen atom compared to tertiary amino groups, a substituent was introduced into the benzyl group to render the proximity of the nitrogen atom sterically congested. The results of this modification of the benzyl group are shown in Table 2. Introduction of a methyl group at the benzyl position of **15** attenuated activity (**16**), whereas *ortho* substitution at the phenyl ring enhanced potency while retaining metabolic stability (**17**). Replacement of the methyl group on the phenyl ring of **17** with a trifluoromethyl group improved stability in rodents (**18**). Encouraged by these results, we investigated halogen substituents at the *ortho* position. In contrast to the disappointing result with the chloro group (**19**), the fluoro group ameliorated inhibitory activity and retained microsomal stability (**20**). Considering the size of a fluoro group, these SAR results might indicate a preference for a small substituent at the *ortho* position. Additional fluorination of the phenyl ring improved microsomal stability in rodents without any loss of activity (**21**, **22** and **23**).

Given this improved metabolic profile in vitro, we evaluated in vivo efficacy of the benzyl derivatives in RANKL-induced osteopenic mice, which were reported as a rapid bone loss model by Yasuda and co-workers.[12] The benzyl compounds **15**, **20, 22** and **23** were given to the mice by daily oral administration for 2 days, concomitantly with intraperitoneal injection of RANKL. In contrast to the insignificant inhibition of **15** at 3 mg/kg, **20, 22** and **23** inhibited bone loss significantly at 3 mg/kg in a dose-dependent manner, owing to the ameliorated metabolic stability and potency in vitro (Fig. 2). Compounds **20** and **23**, in particular, showed significant inhibition at 0.3 mg/kg. In the article of Yasuda et al., special

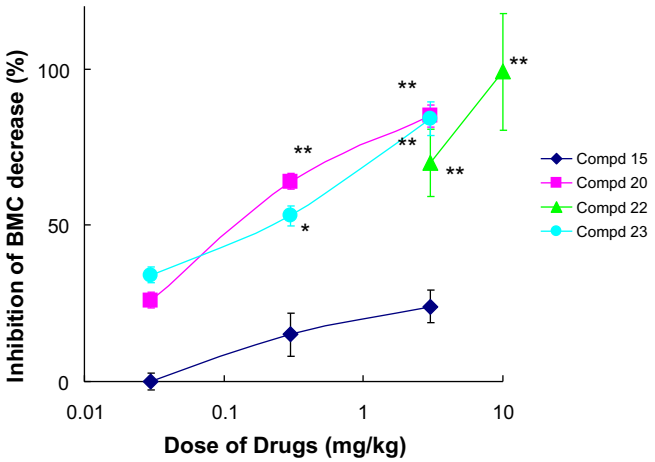
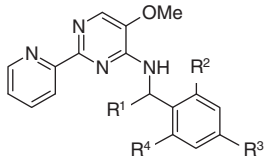


Figure 2. Anti-resorptive effect of compound **15**, **20**, **22** and **23** in RANKL-induced osteopenic mice. Orally administered once daily for 2 days. 10-wk-old female C57BL/6 N mice ($n = 5$). Medium was 0.5% methyl cellulose solution. BMC: Bone mineral content; $^{*}p$ <0.05 versus control; $^{**}p$ <0.01 versus control (Dunnett's multiple comparison test).

protocols were prepared for the assessment of BPs and SERMs; evaluation of BPs required pretreatment with the agents before the first RANKL injection and consequently a longer total duration of the procedures, probably to secure sufficient time to accumulate BPs in bones. Evaluation of raloxifene, a second-generation SERM, was also reported to require ovariectomy after the last RANKL injection, followed by administration of raloxifene for 2 weeks. The efficacy of **20**, **22** and **23** with 2 days administration concomitantly with the RANKL injection supports the idea that the pyridylpyrimidine derivatives have a different mode of action from BPs and SERMs.

The pharmacokinetic study of **20**, **22** and **23**, as described in Table 3, showed increased plasma concentration compared to the HTS hit **1** when administered orally to normal mice. In addition, a single administration of any of these three substituted benzyl compounds exhibited comparable exposure in ovariectomized (OVX) rats, which are the standard model for osteoporosis in postmenopausal women. Furthermore, these three compounds showed an acceptable off-target profile of both CYP inhibition ($IC_{50} \geqslant 5$ μM for 1A2, 2C9, 2C19, 2D6; <10% inhibition of 3A4 at 5 μM for either reversible inhibitory activity or time-dependent inhibitory activity) and hERG inhibition (Rb efflux assay; IC_{50} >25 μM), in addition to good physicochemical properties (solubility in pH6.8 phosphate buffer is >25 μg/ml; PAMPA permeability is $>30 \times 10^{-6}$ cm/s) and toxicological profiles (3T3 NRU phototoxicity test: negative; in vivo micronucleus test: negative).[13]

Table 2
Potency in TRAP staining assay and microsomal stability of 4-benzylaminopyrimidines



Compd	R^1	R^2	R^3	R^4	TRAP staining[a]	$Cl_{int,\ in\ vitro}$[b] (ml/min/kg)		
					IC_{50} (μM)	MLM	RLM	HML
15	H	H	H	H	0.42	911	387	67
16	Me	H	H	H	0.66	570	420	65
17	H	Me	H	H	0.22	901	791	55
18	H	CF_3	H	H	0.23	619	555	77
19	H	Cl	H	H	0.52	704	827	55
20	H	F	H	H	0.13	672	413	61
21	H	F	F	H	0.11	524	N.T.	79
22	H	F	F	F	0.10	486	280	53
23	H	CF_3	F	H	0.19	261	193	77

[a] Inhibitory activity of RANKL-induced osteoclastogenesis using RAW264 cells ($n \geqslant 3$).
[b] Average of two experiments. N.T.: Not tested.

Table 3
Oral pharmacokinetic profiles of compounds **1**, **20**, **22**, and **23** in normal female mice and OVX rats when single dose was administered[a]

Compd	Dose (mg/kg)	Female mouse (10-wk-old C57BL/6N)		OVX rat[b] (12-wk-old Wister)	
		C_{max} (ng/ml)	$AUC_{0-\infty}$ (ng·h/ml)	C_{max} (ng/ml)	$AUC_{0-\infty}$ (ng·h/ml)
1	1	7[c]	15[c]	N.T.	N.T.
20	3	130	240	49	151
22	3	98	188	135	542
23	3	599	1022	156	943

[a] Medium was 0.5% methyl cellulose solution. $n = 3$.
[b] Ovariectomy was conducted 2 weeks prior to administration.
[c] Male mouse. N.T.: Not tested.

In summary, we identified a novel class of RANKL-induced osteoclastogenesis inhibitors using an HTS campaign. Modification of the series to improve metabolic stability led to compounds that are orally bioavailable and efficacious in a murine osteopenia model, with a different mode of action to BPs and SERMs. These compounds showed lower in vitro intrinsic clearance values in human than in rodents. These promising compounds are being evaluated in other bone loss models in rodents, and the results will be published in due course.

Acknowledgments

The authors would like to express their profound gratitude to Mr. Masaharu Yokomoto for his valuable advice and constant support throughout this research. We deeply appreciate Dr. Katsuhiro Yamano, Dr. Hidetsugu Murai, Ms. Masako Furutani, Mr. Fumihiro Nakamori, Dr. Megumi Irie, Dr. Ai Tamura, and Mr. Shoji Takagaki for their measurements in pharmacokinetic studies and valuable discussion. We are grateful to Ms. Miwa Okazawa for her conduct of the HTS, and Mr. Ryutaro Wakayama for his advice on synthesis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.06.087.

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9. Intensity of RANKL signal could be one of factors causing the 10-fold difference in potency of **1** in the two in vitro assays. The promoter assay adopted 50 ng/ml of final RANKL concentration, whereas 8 ng/ml of RANKL was used in the TRAP staining assay.
10. With the same HPLC program, the major metabolites in microsomes were identified as compounds **2** and **3,** which were synthesized as described in the Supplementary data. Metabolites **2** and **3** showed reduced anti-osteoclastogenesis activities in TRAP staining assay, with IC_{50}s of 3.0 and >10 μM, respectively.
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13. Protein kinase panel assay of compounds **22** and **23** showed <40% inhibition against 93 of all tested kinases at 10 μM.