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Identification of novel, exosite-binding matrix metalloproteinase-13 inhibitor scaffolds

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ABSTRACT

Matrix metalloproteinase-13 (MMP-13) has been implicated as the protease responsible for collagen degradation in cartilage during osteoarthritis (OA). Compounds that inhibit the metalloproteinase at the Zn binding site typically lack specificity among MMP family members. Analogs of the low-micromolar lead MMP-13 inhibitor **4**, discovered through high-throughput screening, were synthesized to investigate structure–activity relationships in this inhibitor series. Systematic modifications of **4** led to the discovery of MMP-13 inhibitors **20** and **24** which are more selective than **4** against other MMPs. Compound **20** is also approximately fivefold more potent as an MMP-13 inhibitor than the original HTS-derived lead compound **4**.

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Articular cartilage consists primarily of extracellular matrix (ECM) and a small number of chondrocytes. In turn, cartilage ECM is composed of proteoglycans and collagen, especially fibrillar type II collagen. Type II collagen is extremely resistant to most proteinases because of its triple-helical structure. Several members of the matrix metalloproteinase (MMP) family catalyze the hydrolysis of collagen. MMP-13 is thought to be the most effective enzyme at cleaving type II collagen.¹ Once collagen is cleaved, it denatures at body temperature to become gelatin.² The cartilage degradation and denaturing process is thought to be a primary cause of osteo-arthritis (OA). Thus, the development of a selective small-molecule inhibitor of MMP-13 has great therapeutic potential for halting the progression of OA.

A number of compounds have been described that inhibit MMP-13 and other metalloproteinases by chelating the active site Zn.³ However, these compounds typically display a of lack specificity among MMP family members and have exhibited toxicities in clinical trials, particularly musculoskeletal syndrome (MSS).³

Several selective non-chelating inhibitors of MMP-13 have been reported (for example, compounds **1–3**, Fig. 1).⁴ Molecular modeling and X-ray co-crystal structures indicated that these compounds modulate the activity of MMP-13 by binding to the S1' specificity pocket and preventing substrate from binding. The success of these

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Using high throughput screening (HTS) techniques, we identified several selective low micromolar inhibitors of MMP-13.⁵ One inhibitor (compound **4**; Fig. 2; also known as compound Q in the previous publication, and designated as a MMP-13 probe by the NIH MLPCN program; PubChem CID 2047223) exhibited properties suggesting that its mode of action was not via Zn chelation. Compound **4** possessed a smaller molecular scaffold than previously described MMP-13 inhibitors, yet inhibited only two of the previously tested MMPs, MMP-8 and MMP-13.⁵



Figure 1. Non-chelating inhibitors of MMP-13.

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Figure 2. MMP-13 inhibitor 4 derived from HTS; PubChem CID 2047223.



Scheme 1. General scheme for synthesis of MMP-13 inhibitor analogs of **4**. Reagents and conditions: (a) DBU, MeCN, reflux, or Na, EtOH, $60 \degree$ C, 1-12 h; (b) acetone or DMF, Na₂CO₃, R₃-X.

We pursued a two-pronged approach in our efforts to improve the selectivity and potency of MMP-13 inhibitor **4**. First, structural analogs of **4** were identified by cheminformatic searches using Chemnavigator.com. Analogs containing the thiouracil scaffold with appropriate structural similarity to **4** were purchased from commercial suppliers. Second, analogs of **4** were synthesized starting with the condensation of thiourea (**5**) with a substituted β -ketoester **6** (Scheme 1). Alkylation of the thiocarbonyl unit of **7** with various alkylating agents gave the MMP-13 inhibitor scaffold **8**.

Analogs of compound **4** were initially evaluated for percent inhibition of MMP-13 using a FRET triple-helical peptide substrate assay as described previously.⁵ The triple-helical substrate (fTHP-15) has distinct conformational features that interact with protease secondary binding sites (exosites).^{5,6}

Initial efforts focused on modifications of the cyclopenene ring of **4** (Table 1). These structural changes resulted in a modest

reduction of activity (compounds 10-15) to outright loss of MMP-13 inhibition (compounds 9 and 16-19). Replacement of the cyclopentenyl group by a propyl group in **9** resulted in loss of all MMP-13 inhibitory activity; however, analog 10 with a methyl group at this position retained modest activity. An analog with methyl groups at both available positions on the uracil ring (compound 35, Table 2) also displayed excellent MMP-13 inhibition activity. Amine and sulfonamide substituted analogs 11 and 12 retained modest MMP-13 inhibition, as did the fused thiophene derivative 15. However, incorporation of a fused phenyl ring (compound 16), a cyclohexyl (see 34 in Table 2) or a fused pyrazole ring (compound 17) resulted in inactive compounds. Attempts to extend substituents in compound 14 into regions of space that the side chains of 1-3 might occupy (assuming that 4 were to bind in the same site as 1-3 led to decreased activity compared to 4. while compounds 18 and 19 were inactive.

While the data in Table 1 suggested that modifications of the cyclopentenyl ring of 4 did not lead to increased MMP-13 inhibition, encouraging results emerged for two compounds (20 and 24) in the group of analogs presented in Table 2 with differentially substituted benzylic groups. The inhibition data for analogs 20 to 24 and 35 demonstrate that a variety of para substituents on the benzylic unit are compatible with MMP-13 inhibition, and that a meta substituent leads to decreased activity (compare 20 and 21). The carboxylic acid 25 suffered a severe loss of inhibition in comparison with the methyl ester 24, suggesting that a very polar or charged substituent is not permitted in this segment of the inhibitor binding pocket. Replacement of the benzylic aryl group with alkyl, alkenyl (an analog with $R = H_2C = CHCH_2CH_2CH_2CH_2S =$ analogous to 29 was inactive), and alkynyl chains (28, 29), or extending the alkyl chain between the sulfur and the aryl group (26, 27) proved detrimental to MMP-13 inhibitory activity. Attempts to introduce other polar or hydrogen-bonding substituents, as in compounds 30, 31, 32, and 33, also led to loss of activity in this inhibitor series.

The four most potent MMP-13 inhibitors in this structural series (**4**, **20**, **24**, and **35**) have *para* substituents on the benzylic side chain. The only deviation from the original heterocyclic scaffold that did not significantly lessen inhibition was the exchange of the cyclopentene-fused uracil ring system of **4** with the dimethyl substitute uracil in **35**. Counter screening of these compounds

Table 1

Inhibition of MMP-13 activity by analogs of compound 4 with replacement of the original cyclopentenyl-fused uracil



All structures in this table have the chlorobenzyl substituent of **4**. Assays were performed in triplicate using 40 µM of each compound and fTHP-15 as substrate as described.⁵ The standard deviations were <10%.





Assays were performed in triplicate using 40 µM of each compound and fTHP-15 as substrate as described.⁵ The standard deviations were <10%.

was performed against five other members of the MMP family, specifically MMP-1, MMP-2, MMP-3, MMP-8, and MMP-14/MT1-MMP, to evaluate their MMP selectivities. Since inhibition of MMP-13 collagenolytic activity is desired for OA applications. counter screening of other collagenolytic MMPs (MMP-1, MMP-2, MMP-8, and MMP-14) was pursued, along with a non-collagenolytic MMP (MMP-3) as a control. Due to its relatively high activity against MMP-8 (20% inhibition at 40 µM; data not shown), compound 35 was not further examined. The only MMP that was inhibited significantly by all three compounds was MMP-13 (Table 3). Compounds 20 and 24 were better inhibitors of MMP-13 than the original inhibitor 4. Compound 20 did not inhibit MMP-1 or MMP-8, and showed low levels of inhibition of MMP-2, MMP-9, and MMP-14 (Table 3). This is distinct from compound 4, which inhibited MMP-8. Compound 24 was less selective than compound 20, inhibiting MMP-8 and showing greater levels of inhibition of MMP-14 (Table 3).

Single inhibitor kinetic assays were performed with MMP-13 and fTHP-15 using methods described previously^{5,7} to determine the inhibition constant (K_i) and modality of compounds **20** and **24**. Lines of best fit crossing on the X axis indicated a non-competitive mode of inhibition of MMP-13-mediated hydrolysis of the fTHP by compound **20** (Fig. 3, left). The K_i value was calculated to be 824 ± 171 nM. Lines of best fit crossing on the X axis also indicated a non-competitive mode of inhibition of MMP-13-mediated hydrolysis of the fTHP by compound **24** (Fig. 3, right). The K_i value was calculated to be 1526 ± 260 nM. The K_i value for compound **4** was 3800 nM (Table 4).

An RP-HPLC-based secondary screen was performed to determine whether the compounds of interest were artifactual inhibitors of MMP-13.⁵ Hydrolyzed substrate was examined before and after inhibitor addition to evaluate fluorescence interference. Compounds **20** and **24** were inhibitors in the RP-HPLC secondary screen Table 3

Inhibition of MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, and MMP-14 activity by compounds 4, 20, and 24

Target	Inhibition (%) at [inhibitor] = 40 µM		
	4	20	24
MMP-1	0	0	0
MMP-2	25 ± 11	19 ± 2.1	26 ± 9.2
MMP-8	17 ± 3.0	0	8.0 ± 5.0
MMP-9	10 ± 4.0	7.0 ± 3.0	16 ± 4.0
MMP-13	51 ± 0	67 ± 2.0	61 ± 1.0
MMP-14	22 ± 5.0	20 ± 6.0	31 ± 9.0

Assays were performed using 40 μ M of each compound and fTHP-15 as substrate as described.⁵ Enzyme concentrations were 10 nM for MMP-2, MMP-9, and MMP-14, 2.3 nM for MMP-13, 4 nM for MMP-1, and 2 nM for MMP-8.

(data not shown). Thus, the results from the FRET assay (Table 3) were not due to fluorescence artifacts.

The identification of a selective, non-competitive MMP-13 inhibitor (compound 4) led to the present medicinal chemistry characterization of the 2-(arylmethylthio)-cyclopentapyrimidin-4-one scaffold. Two compounds (2-[(4-methylphenyl)methyl sulfanyl]-1,5,6,7-tetrahydrocyclopenta[*d*]pyrimidin-4-one; **20** and methyl 4-[(4-oxo-1,5,6,7-tetrahydrocyclo penta[d]pyrimidin-2yl)sulfanylmethyl] benzoate; 24) demonstrated improved potency (as measured by K_i) and selectivity compared to the previously reported compound 4. Most significantly, compound 20 did not inhibit MMP-8, whereas compound 4 did. The lack of inhibition of MMP-8 is desirable, as MMP-8 is a prominent anti-target.⁸ Additionally, MSS has been attributed to inhibition of MMP-1 and ADAM17/TACE,9 and compounds 4, 20, and 24 do not inhibit MMP-1 (Table 3) or TACE (data not shown). Initial clinical trials with MMP inhibitors were disappointing, with one of the problematic features being a lack of selectivity.^{3b,10} Compounds **20** and **24**



Figure 3. Lineweaver-Burk plot of MMP-13 inhibition of fTHP-15 hydrolysis by compound 20 (left) or 24 (right).

Table 4 IC $_{50}$ or K_i values (µM) for inhibition of MMP-1, MMP-8, and MMP-13 by compounds 4, 20, and 24

Target	Inhibitor		
	4	20	24
MMP-1 (IC ₅₀)	>40	>40	>40
MMP-8 (IC ₅₀)	>40	>40	>40
MMP-13 (K _i)	3.8	0.8	1.5

are selective exosite-binding inhibitors of MMP-13, and thus represent leads for the development of the next generation in metalloproteinase therapeutics.

We initially assumed that compound 4 would bind within the same S1' specificity pocket as compound 1, and designed multiple analogs to achieve interactions present in the crystal structure of compound **1** bound to MMP-13.^{4e} However, because none of these changes led to substantially improved activity in the compound 4 analogs, we suspected that compound 4 and its analogs did not bind in the S1' pocket as did compound 1. Preliminary hydrogen-deuterium exchange mass spectrometry experiments⁶ found protection of MMP-13 from exchange by compound 1 at the previously observed site of compound **1** binding,^{4e} but no protection from exchange by compound 20 at this site (M. Chalmers, P. Griffin, P. Hodder, G. Fields, and W. R. Roush, unpublished results). Dual inhibition kinetic assays^{4f} demonstrated non-mutually exclusive binding between compound 4 and either compound 1 or 3 (D. Minond, T. P. Spicer, W. R. Roush, G. B. Fields, and P. S. Hodder, manuscript in preparation). We are pursuing detailed biochemical characterization and competition experiments to further evaluate the unique binding of compound 4 and its analogs, and will report those results in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.077.

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