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 5-fold more potent as an MMP-13 inhibitor than the original HTS-derived lead compound **4**.

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 osteoarthritis (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, 2** and **3**, Figure 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 compounds suggest that, by avoiding direct Zn interactions, one might be able to achieve inhibitor specificity among metalloproteinase subtypes and related enzyme families and thereby reduce toxicity.

Using high throughput screening (HTS) techniques, we identified several selective low micromolar inhibitors of MMP-13.[5] One inhibitor (compound **4**; Figure 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]

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 to give 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* subsituents on the benzylic side chain. The only deviation from the orginal 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 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** (Figure 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** (Figure 3, right). The K_i value was calculated to be $1,526 \pm 260$ nM. The K_i value for compound **4** was 3,800 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 (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 compound**s** (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-2-yl)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** 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 experiments[6] 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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7. K_i values were determined by non-linear regression (hyperbolic equation) analysis using the mixed inhibition model which allows for simultaneous determination of the mechanism of inhibition.[10] The mechanism of inhibition was determined using the "alpha" parameter derived from the mixed-model inhibition by GraphPad Prism. The mechanism of inhibition was additionally confirmed by Lineweaver-Burk plots (Figure 3).

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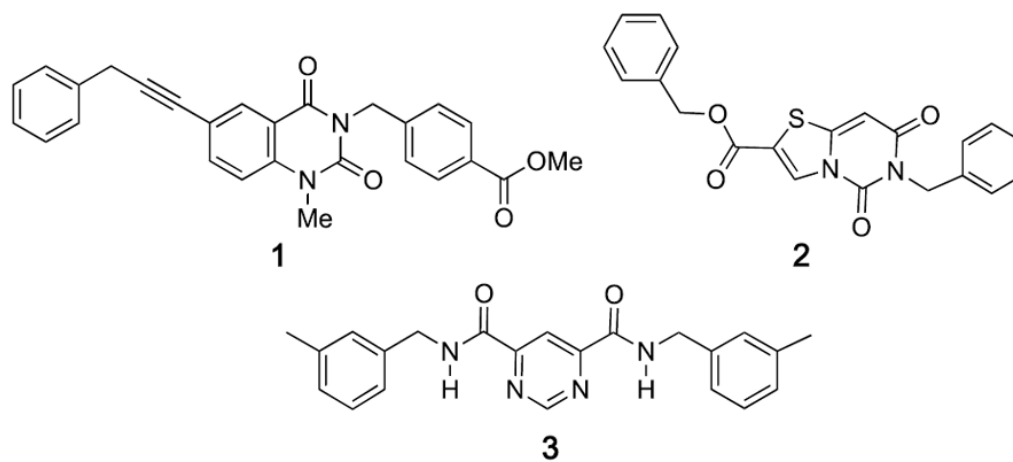10. Overall CM, Kleifeld O. Br J Cancer. 2006; 94:941. [PubMed: 16538215]
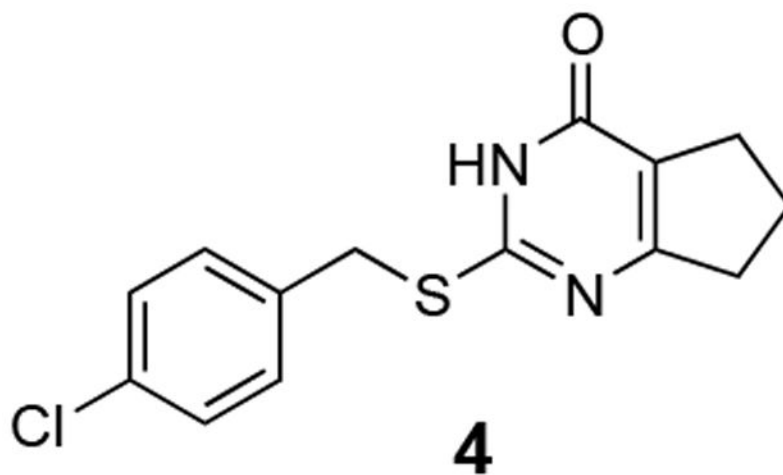
Figure 1.
Non-chelating inhibitors of MMP-13.

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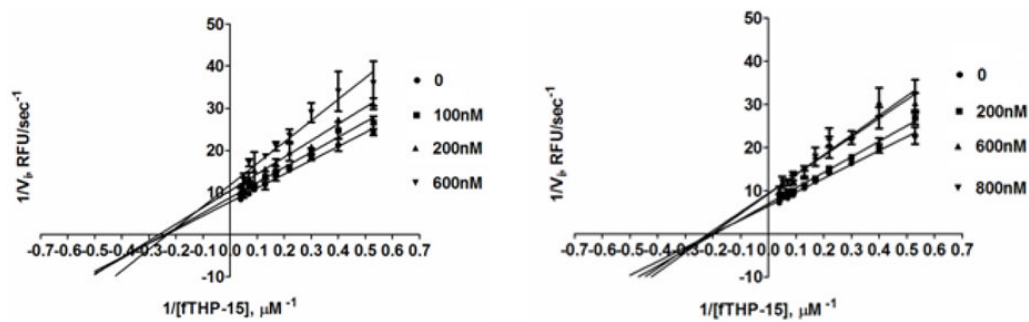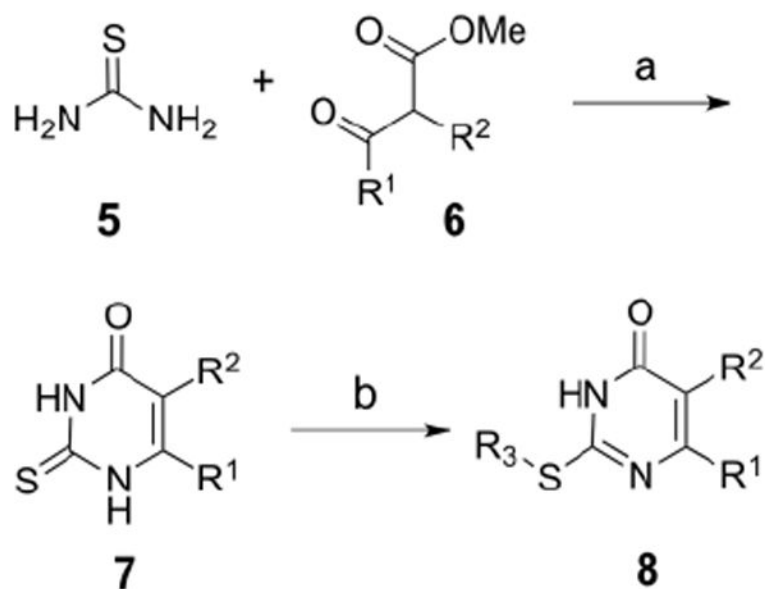MMP-13 inhibitor **4** derived from HTS; PubChem CID 2047223

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

Scheme 1.
General scheme for synthesis of MMP-13 inhibitor analogs of **4**: (a) DBU, MeCN, reflux, or Na, EtOH, 60 °C, 1-12 h. (b) acetone or DMF, Na_2CO_3, R_3-X.

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%.

Compound	MMP-13 % Inhibition	Compound	MMP-13 % Inhibition	Compound	MMP-13 % Inhibition
4	49.1	**12**	31.2	**16**	inactive
9	inactive	**13**	30.5	**17**	inactive
10	32.9	**14**	37.7	**18**	inactive
11	37	**15**	36.6	**19**	inactive

Table 2

Inhibition of MMP-13 activity by analogs of compound 4 with different S-alkyl substituents

Assays were performed in triplicate using 40 μM of each compound and fTHP-15 as substrate as described.[5] The standard deviations were <10%.
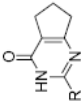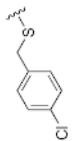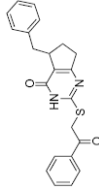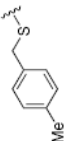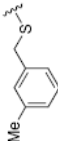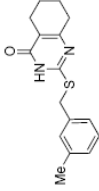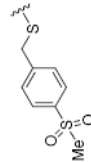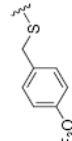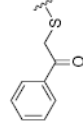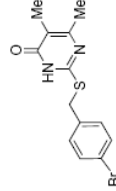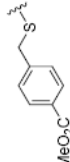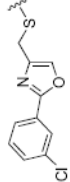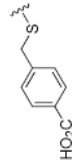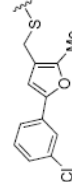
	Compound, R =	MMP-13 % Inhibition		Compound, R =	MMP-13 % Inhibition		Additional Analogs	MMP-13 % Inhibition
4		49.1	26		7.7	33		16.0
20		68.7	27		7.2	34		inactive
21		45.2	28		10.3	35		55.8
22		46.7	29		17.1			
23		34.4	30		inactive			
24		62.5	31		inactive			
25		2.2	32		Inactive			

Table 3

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

Assays were performed using 40 μM 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.

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

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