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N-Hydroxyformamide Peptidomimetics as TACE/Matrix Metalloprotease Inhibitors: Oral Activity via P1′ Isobutyl Substitution

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Abstract—*N*-Hydroxyformamide-class metalloprotease inhibitors were designed and synthesized, which have potent broad-spectrum activity versus matrix metalloproteases and TNF-α converting enzyme (TACE). Compound **13c** possesses good oral and intravenous pharmacokinetics in the rat and dog.

The matrix metalloprotease (MMP) family of endopeptidases represents a group of tightly regulated metalloproteases mediating turnover of the extracellular matrix proteins proteoglycan, collagen, and gelatin.[1] Increased levels of certain MMPs are evident in rheumatoid arthritis and osteoarthritis.[2] MMPs are also present in certain cancers at the tumor epithelium, and are implicated in metastasis.[3] Given the extent of disease association with MMPs, the pharmaceutical industry has dedicated substantial resources to MMP inhibitor discovery with agents at all stages of development.[4]

Tumor necrosis factor-α (TNF-α) is a cytokine produced by many cell types but mainly by those of monocytic lineage. Elevated TNF levels are implicated in the pathologies of rheumatoid arthritis,[5] multiple sclerosis,[6] type II diabetes,[7] inflammatory bowel disease,[8] and other human ailments. TNF-α is unusual among the cytokines in that it is processed from a 26 kD, membrane-bound form to a 17 kD soluble form by a specific proteolytic cleavage. A subset of known matrix metalloprotease inhibitors was subsequently shown to inhibit this processing event in cells,[9–11] and subsequently the enzyme activity was characterized, cloned, and expressed.[12] TNF-α converting enzyme (TACE, or ADAM17) is a member of the ADAM family of metalloproteases, a rapidly expanding family of metalloproteases implicated in diverse biological function. Therefore, TACE/MMP inhibitors have been and continue to be of significant interest in the biomedical community. The debate continues to be centered on the desired selectivity profile of metalloprotease inhibitors. The recent clinical successes of biologicals, such as etanercept[13] and infliximab[14] that neutralize TNF suggest a selective TACE inhibitor would be preferred. While incorporating collagenase and/or aggrecanase inhibitory activity seems to be a

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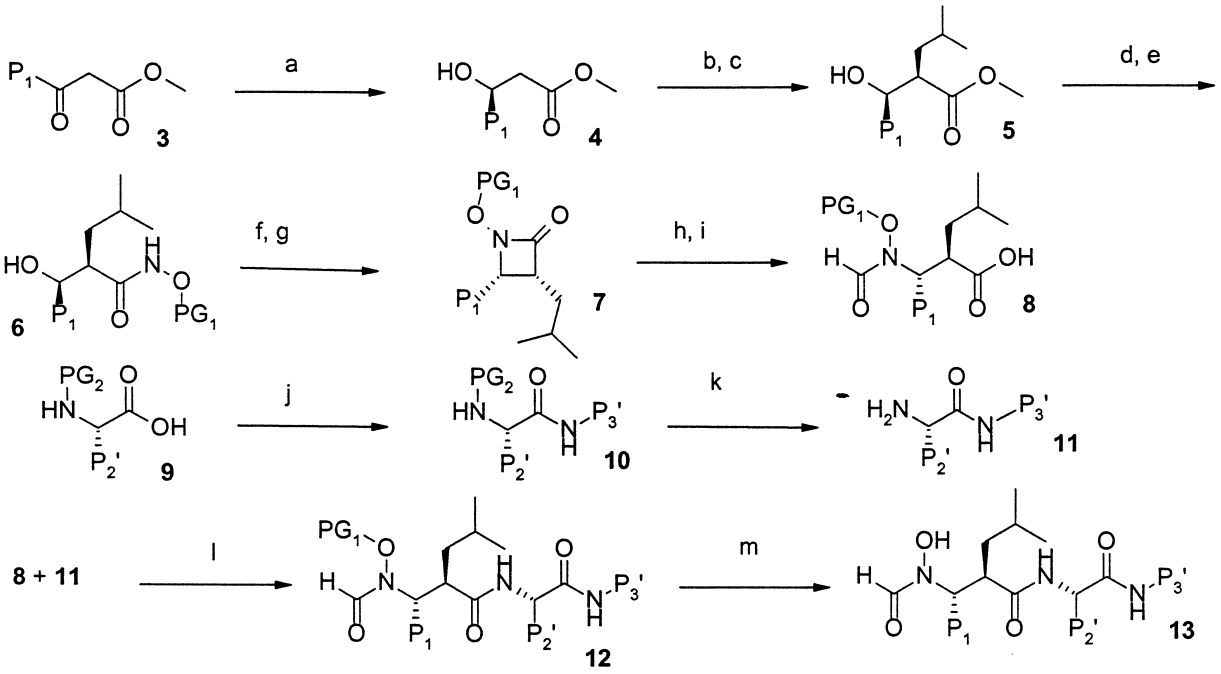

Scheme 1. (a) $[Ru(R\text{-}(+)\text{-BINAP})Cl_2]_2NEt_3$, catalytic HCl, MeOH, H_2, 60 psi; (b) LDA, THF; methallyl bromide, HMPA, −78–0 °C; (c) H_2, Pd/C, MeOH, 50 psi; (d) NaOH, H_2O, MeOH; (e) 1-ethyl-3-(3-dimethylamino propyl)carbodiimide, CH_2Cl_2, *O*-(2-tetrahydropyranyl)hydroxylamine, 0–25 °C; (f) methanesulfonyl chloride, CH_2Cl_2–pyridine, 0–25 °C; (g) K_2CO_3, acetone, reflux; (h) NaOH, H_2O; (i) formic acetic anhydride, CH_2Cl_2–pyridine, 0–25 °C; (j) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 1-hydroxybenzotriazole, *N*-methylmorpholine, DMF, P3′-NH_2; (k) 4 N HCl/dioxane, CH_2Cl_2 (for PG_2 = *tert*-butoxycarbonyl) or H_2, Pd/C (for PG_2 = benzyloxycarbonyl); (l) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 1-hydroxybenzotriazole, *N*-methylmorpholine, DMF; (m) acetic acid, 25 °C, 3 days, or 50 °C, 6–10 h for (PG_1 = 2-tetrahydropyranyl) or H_2, Pd/C (for PG_1 = benzyl).

logical tactic to preserve the joint cartilage, this approach to inhibitor development may increase the likelihood of adverse events. The broad-spectrum matrix metalloprotease inhibitor marimistat[3] has been reported to cause musculoskeletal pain or 'tendonitits' in cancer patients. While the precise mechanism behind this clinical observation is not understood, more specific inhibitors should allow us to better understand the pharmacology observed in the clinic.

Structure–activity relationships for MMP inhibitors such as Ro31-9790[15] (**1**) constructed on a tripeptidomimetic 'right-side' template possessing a hydroxamate or carboxylate active-site zinc chelator are documented (Fig. 1), but less well known are the analogous right-side templates incorporating an *N*-hydroxyformamide chelator (**2**). *N*-Hydroxyformamide chelators have been explored for ACE/NEP inhibition with some success.[16] These 'retrohydroxamates' were seen to offer potential advantage in drug properties, since they might possess lower intrinsic hydrophilicity as well as less propensity for biological conjugation or hydrolysis relative to hydroxamic acids. Described herein are some initial efforts at synthesis of P1′ isobutyl *N*-hydroxyformamide tripeptidomimetics and determination of their potential as MMP/TACE inhibitors in vitro and in vivo.

The synthetic route to compounds of general structure **13** is shown in Scheme 1. Asymmetric reduction of a β-ketoester[17] **3** is followed by alkylation of the derived alkoxy enolate with methallyl bromide.[18] Catalytic hydrogenation of the alkylation product affords the ester **5**. Saponification of **5** to the hydroxy acid followed by coupling with 2-tetrahydropyranyloxyamine or *O*-benzylhydroxylamine hydrochloride gives the protected *N*-hydroxyamide **6**. Methanesulfonylation of the hydroxyl group of **6** followed by addition of the mesylate to refluxing potassium carbonate in acetone affords the β-lactam **7**.[19] Alternately, the transformation of **6** to **7** can be accomplished in one step via cyclodehydration with diisopropylazodicarboxylate/triphenylphosphine in tetrahydrofuran at 0 °C. Hydrolysis of the β-lactam **7** is followed by *N*-formylation to give the formamido acid **8**. A suitably *N*-protected α-amino acid **9** is treated with

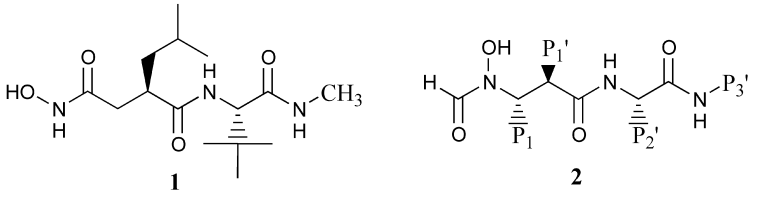

Figure 1.

Table 1. In vitro and in vivo activitites of compounds **1** and **13a–k**

Entry	P_1	$P_{2'}$	P_3	Cell TNFα Inhibition, MonoMac-6 (IC_{50}, nM)	TACE K_i (nM)	MMP1 K_i (nM)	MMP9 K_i (nM)	MMP3 K_i (nM)	Murine LPS-Induced Plasma TNF (% inhib. po)
1	—	—	—	5716	87	2	2	310	Inactive[a]
13a	CH_3		H	3073	156	181	33	406	nd[b]
13b	CH_3		CH3	2954	142	17	21	2323	nd
13c	CH_3			245	20	27	34	437	60
13d	CH_3			270	20	41	29	93	48
13e	CH_3			681	27	36	17	120	61
13f	CH_3			3964	64	170	98	354	Inactive
13g	CH_3			1686	55	97	17	80	Inactive
13h	$-C_2H_5$			200	16	16	28	135	52
13i	$-C_2H_5$			304	29	47	89	153	61
13j	$-C_2H_5$			92	11	42	24	45	48
13k	$-C_2H_5$			1414	31	55	73	38	62

[a]Inactive means no effect on TNF levels.
[b]nd, not determined.

Table 2. [24]Pharmacokinetic data for **13c**

Species	Dose iv (mg/kg)	C_{max} iv (ng/mL)	AUC iv (h*ng/mL)	$t_{1/2}$ iv (h)	Cl(s) iv (mL/min/kg)	F (%)
Rat	3.4	2330	957	3.4	62	26
Dog	1.2	4530	2100	2.8	10	44

water soluble carbodiimide and *N*-hydroxybenzotriazole and a substituted amine to afford the amide **10**, which is deprotected to give the amino amide **11**. Coupling of the amino amide **11** and the acid **8** provides the protected hydroxyformamide **12**. Acidic media (for PG_1 = 2-tetrahydropyranyl) or hydrogenolytic (for PG_1 = benzyl) deprotection of **12** followed by solvent removal and precipitation or crystallization affords the product metalloprotease inhibitor **13**.

Compounds were tested for inhibition of cell-free TACE according to a previously published protocol.[20] Compounds were evaluated also for inhibition of collagenase-1 (MMP1), gelatinase B (MMP9, 92 kD gelatinase), and stromelysin-1 (MMP3) according to a standard protocol.[21] Compounds were also evaluated for inhibition of cellular release of 17 kD TNF-α from MonoMac-6 cells according to a standard protocol.[22] Lastly, compounds were assessed for in vivo inhibition of plasma TNF-α upon oral administration at 40 mg/kg, in lipopolysaccharide (LPS) treated mice according to a standard protocol.[23]

The hydroxamate Ro31-9790 **1** inhibits TACE with respectable potency and is more potent versus the MMPs. The *N*-hydroxyformamide carboxamide and *N*-

methylamide **13a** and **13b** are weaker MMP inhibitors but comparable in potency versus TACE. All three molecules lack potency in cells, however. The addition of a heteroaryl substituent at P3′ (**13c**) engenders TACE potency in vitro and in cells, and preserves respectable potency versus MMPs.

Given heteroaryl substitution at P3′, some trends are evident from the data in Table 1. TACE and MMP potency changes little as P1 is changed from methyl to ethyl, as cell potency tends to increase (entries **13c**/**h**, **13d**/**j**). Increased lipophilicity at this place in the molecule might tend to shield the polar *N*-hydroxyformamide substituent from hydration and thereby promote enzyme binding or partitioning into membrane lipid. Thiazole at P3′ engenders better TACE and cell potency, with little effect otherwise (entries **13h**/**i**, **13j**/**k**). Butyl isomers at P2′ given show uniform potency versus TACE, but cell potency varies with the nature of branching. *tert*-Butyl and *s*-butyl perform best in the cell assay, while isobutyl does not. The analogous isopropyl compound **13e** is good in the cell-based assay while the neopentyl compound **13f** is poor. Clearly branching at the P2′ position vicinal to the peptide chain is essential for good cell-based activity. Further, such branching promotes oral activity in the murine LPS-induced TNF assay, as all P3′ heteroaryl compounds except **13f** and **13g** show oral activity in this model.

Compound **13c** was further evaluated for its pharmacokinetic properties in rat and dog (Table 2).[24] Upon intravenous and oral dosing in either species, **13c** demonstrates good half-life and bioavailability. The addition of a P3′ heteroaryl substituent to the *N*-hydroxyformamide peptidomimetic structure **2** has afforded potent inhibitors of TACE and MMPs. The nature of the P2′ and P1 substituents directly influences TNF inhibition in cells and in the mouse in vivo. Compound **13c** could form the basis for a TNF-α targeted drug therapy acting via inhibition of TACE and further studies will be reported in due course.

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21. Recombinant catalytic domains of MMP-1 and full length active MMP-3 were expressed and purified from *Escherichia coli*. Enzymes were refolded in 200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 10 μM $ZnSO_4$ and 0.01% Brij 35, pH 7.6 for 1 h prior to the assay. The catalytic domains of proMMP-9 were purified from the media of baculovirus infected *T. ni* cells. Assays were run in a total volume of 0.180 mL assay buffer containing 200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 10 μM $ZnSO_4$ and 0.01% Brij 35, pH 7.6. MMP-1, MMP-3, and MMP-9 concentrations were adjusted to 0.5, 0.05, 5, and 0.1 nM, respectively. Enzymes were pre-incubated with inhibitor for 20 min at room temperature and the reactions were initiated with the addition of the fluorogenic substrate, Dnp-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Nma)–NH_2. Dose responses were generated using 11 point 3-fold dilutions of the inhibitor. Product was measured using excitation and emission wavelenghts of 343 and 450 nm, respectively.

22. Human mono mac-6 cells in RPMI 1640 media with 10% fetal bovine serum (FBS) were preincubated for 10 min with compounds and then stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (Sigma, #P-8139) and 30 ng/mL LPS (Sigma, #L2630) and TNF measured in the media at 2 h by ELISA kit (R&D Systems, Minneapolis, MN, USA, #DTA50).
23. Test compounds are formulated in 0.2 mL of PBS and 0.1% Tween 80 and given orally via gavage 10 min prior to LPS administration. C3/hen female mice are injected intraperitoneally with 200 μg/kg LPS (*E. coli*, Serotype 0111:B4, Sigma Chemical Co, St. Louis, MO, USA) in PBS and sacrificed 90 min later by CO_2 asphyxiation. Blood is immediately taken from the caudal vena cava and plasma prepared and frozen at −80 °C. Plasma concentrations of TNF are measured by ELISA (Genzyme Co., Cambridge MA, USA).
24. Compound **13c** was formulated in a 2.5–5% (w/v) lecithin/water emulsion and administered iv via a cannula or orally via a feeding tube to male Lewis rats or Beagle dogs. Blood was collected at selected timepoints over a 24 h period following dosing. Plasma was prepared and then treated with at least two volumes of methanol or acetonitrile to remove plasma protein. The extracts were subsequently assayed for compound **13c** using HPLC/MS. The area under the plasma concentration versus time curve (AUC) was determined by the linear trapezoidal method and includes the area resulting from extrapolation along the elimination phase to time infinity. Oral bioavailability (F, %) was determined from the ratio of AUCoral to AUCiv. A crossover design was used for dog studies such that iv and oral doses were administered to the same animal with a week between dosings in order to allow for compound washout. Values are means of at least two animals.