MMP inhibition by barbiturate homodimers

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Abstract

We have studied some homodimeric compounds derived from 5-homopiperazine substituted pyrimidine triones (barbiturates) with linkers in the range 2–20 carbon atoms. The compounds were designed to be capable of resisting absorption, to be stable in the gut and to maintain inhibitory potency against gelatinases and related function.

The compounds were then assessed for inhibitory potency against a panel of MMPs (1, 2, 8, 9 and 13). The dimer compounds had similar potency and selectivity to the homopiperazine barbiturate monomer class. At 100 nM, selected dimers significantly inhibited cancer cell invasion in a matrigel assay using Caco-2 cells stimulated by hepatic growth factor. Finally, selected dimers showed adequate stability in simulated intestinal fluid to suggest the capacity to transit to the colon.

Matrix metalloproteinases (MMPs) are a group of essential zinc dependent proteolytic enzymes that become dysregulated in several disease processes such as inflammatory states and cancer.[1–3] The MMPs are long-standing drug development targets with notoriously low levels of clinical translation of inhibitors due both to lack of efficacy and side effects in clinical trials. The failure of successive MMP inhibitors is frequently explained by the inadequate selectivity of test compounds.[4,5] Selectivity in this homologous family is indeed a great challenge, but it is not the only one. There is increasing evidence that MMP inhibition has the capacity to provoke homeostatic responses within local proteolytic networks that can oppose the therapeutic goal (this is very well reviewed in Ref. 6). The failure of genetic ablation studies to establish unambiguous pathophysiological roles for individual MMPs is at least consistent with this. Specific MMPs may play disease promoting roles in specific tissue and possibly only at specific times within the disease life-cycle.[6,7]

Two MMPs, the so-called gelatinases, MMP-2 and MMP-9, are associated with inflammatory and neoplastic diseases of the intestinal tract. Thus, levels of MMP-2 and MMP-9 are increased in colorectal tumours compared to normal mucosa.[8,9] The latent MMP-2, but little or no active MMP-2, has been found in healthy mucosa, but the active MMP-2 is significantly increased in flat-depressed adenomas with invasiveness capacity and colorectal cancer specimens.[10,11] By contrast, up-regulation of pro-MMP-9 is an early event in the colorectal adenoma-carcinoma sequence.[11,12]

We reasoned that the disposition of the gelatinases in the intestinal tract and their putative involvement in intestinal disease processes suggests that non-absorbable inhibitors might have a disease modifying effect. The properties of a compound that promote or inhibit its enteral absorption are well known and a non-absorbable strategy has been adopted successfully in several other therapeutic areas where confinement within the GIT is considered desirable.[13]

We have recently reported on *N*-acyl homo-piperazine substituted pyrmidine triones that have excellent in vitro potency for the gelatinases.[14] Since the barbiturates, along with other Zn binding inhibitors of the MMPs, bind on the surface of the enzyme, we hypothesized that the barbiturate homo-dimers might retain inhibitory properties of the monomers but have reduced cell penetration due to their mass. In this paper we present preliminary results showing that the dimeric barbiturates are endowed with a number of properties important for drug development, that is, exert good MMP inhibitory activity, they significantly inhibit colorectal cancer cell invasion (CACO-2) in a model of cancer cell invasion while they retain adequate stability in intestinal conditions to transit the intestinal tract.

The dimer compounds **2**–**9** were synthesized by coupling of **1** (available from our previous work) with the appropriate acid dichlorides at low temperature (−70 °C)[15] (Scheme 1). A further three dimers (**10**–**12**) were prepared by adding dicarboxylic acids to a mixture of **1**, DCC, and DMAP in THF at room temperature for 24 h. The reaction mixtures were dried in vacuo to remove the solvent. Test articles were chromatographically pure (HPLC) and characterized by NMR and MS. In order to examine the effects of the linker chemistry on inhibitory potency and selectivity a further two *N*-acyl compounds were prepared but without the second inhibitory unit. The butyrate and myristate monomers **13**, **14** were prepared by treating the amine with the relevant carboxylic acid in the presence of DCC/DMAP.

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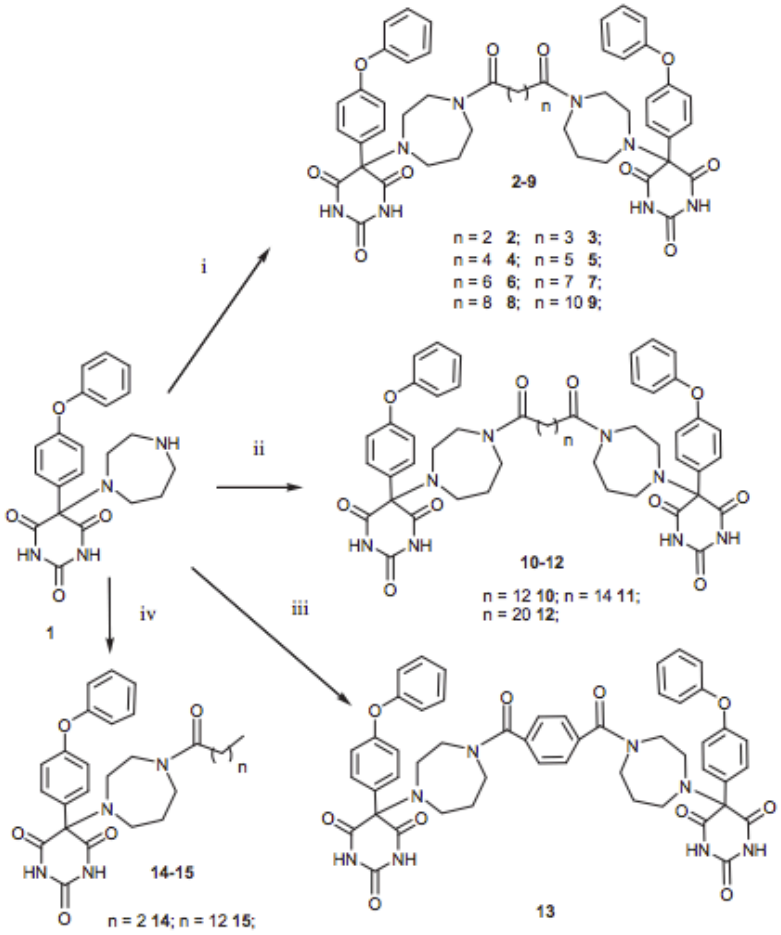

Scheme 1. (i) Acid dichlorides, triethylamine, THF, −70 °C, 8 h, 20–31%; (ii) DCC, DMAP, diacids, THF, rt, 24 h, 17–27%; (iii) terephthaloyl dichloride triethylamine, THF, −70 °C, 8 h, 24%; (iv) butyryic or myrstic acid, DCC, DMAP, THF, 24 h, 25–45%.

The inhibitory effects of the dimer series on MMP-2, -9, -8 and -13 activity were measured using a fluorogenic assay with human recombinant enzymes. The compounds were tested at 0.01–10,000 nM following incubation for 45 min. The compounds did not exhibit significant activity when screened against MMP-1 at 10 μM, an expected consequence of their phenoxyphenyl P1′ substitution.

All dimers exhibited IC_{50} values in the nanomolar range. Compounds **2**, **6**, **7**, and **13**, resulted in IC_{50} values <100 nM (Table 1).

Table 1
IC_{50} values (nM) for inhibition of MMPs ($n = 3$ over five concentration levels)

Cpd	MMP-2	MMP-9	MMP-8	MMP-13
2	57 (48–67)	85 (66–108)	44 (30–64)	11 (8.0–16)
3	54 (42–71)	188 (153–232)	85 (62–117)	164 (117–229)
4	149 (122–182)	115 (91–146)	60 (42–85)	23 (18–28)
5	82 (66–101)	121 (98–149)	117 (90–151)	648 (53–78)
6	67 (58–79)	80 (70–92)	88 (62–123)	155 (121– 200)
7	56 (48–65)	71 (62–81)	98 (81–118)	44 (36–54)
8	69 (54–88)	131 (106–162)	91 (75–111)	106 (86–132)
9	145 (91–231)	120 (82–175)	125 (80–196)	104 (82–130)
10	227 (159–323)	81 (159–323)	217 (142–333)	70 (38–127)
11	52 (35–78)	222 (150–330)	282 (221–361)	134(82–129)
12	71 (44–115)	144 (103–201)	162 (129–203)	225 (159–318)
13	54 (40–74)	50 (40–64)	46 (34–64)	23 (19–27)
14	21 (16–27)	13 (9.5–18.6)		
15	598 (446–805)	608 (408–905)		

The most MMP-2 selective compound was **11** (IC_{50}: MMP-2 = 52 nM, MMP-9 = 222 nM), whereas **10** showed the best MMP-9 selectivity. The butyric acid monomeric amide **14** was the most potent compound tested (IC_{50} 21, 13 nM, MMP2/9). Gelatinase inhibitory potency in the monomeric *N*-acyl substituted homopiperazine class is influenced by the *N*-acyl substituent.[14] The result for amide **14** is consistent with earlier observations. The gelatinases are intolerant of binding modes requiring placement of rigid barbiturate substituents at the S2′ pocket but they are tolerant of a variety of flexible side chain groups-highest potency has been observed in compounds with relatively small P2′ substituents. On the other hand, the longer chain monomer compound **15**, a myristic acid amide was the least potent compound in this study (IC_{50} 600 nM), being 5–10 fold lower in potency than the dimers of comparable chain length (**9**–**12**).

Figure 2 shows a representative conformation following docking experiments (AutoDock 4) with **3** using the X-ray crystal structure of a mutant MMP-9 bound to a 5-piperazinyl barbiturate (PDB code 2OVX). Figure 2 shows the barbiturate in the expected Zn binding mode with the phenoxyphenyl group penetrating the deep hydrophobic P1′ pocket, the homopiperazine directed into the P2′ pocket, away from the enzyme and the second Zn binding unit bound on the enzyme surface.

The gelatinases are found in various monomeric and oligomeric states. MMP-2 binds to TIMP-2, -3, -4 and interacts with glycosaminoglycans. The activation of MMP-2 is unique among MMPs

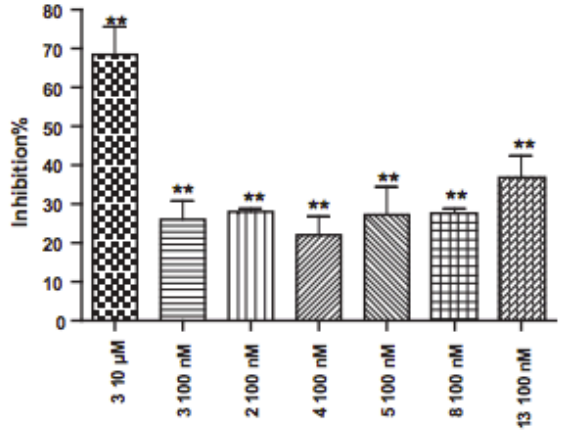

Figure 1. Inhibition of Caco-2 cell invasion by barbiturate dimer analogs (**significantly different from control $P < 0.01$). Data were analyzed using one-way analysis of variance (GraphPad Prism 3, San Diego, CA, USA). The results are expressed as mean ± SEM of at least three independent experiments.

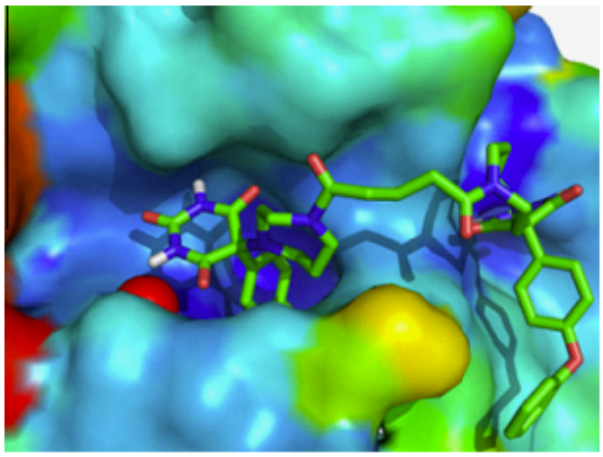

Figure 2. A picture of **3** docking with a 'Connolly' surfaces on MMP-9. The surfaces were generated by the programme PyMOL® based on the crystal structures of MMP-9 (PDB code 2ovx). Ligand orientations were used which had the lowest binding energy of the conformations of 50 runs with 25,000,000 energy evaluations in Autodock4 and displayed by PyMOL®. The active site zinc atoms are shown as the red spheres.

as it needs the formation of the trimeric activator complex proMMP-2/TIMP2/MT1-MMP).[16] MMP-9 is secreted in monomeric and stable homodimeric forms. It is the only secreted MMP to form homodimers but it is relatively promiscuous, forming complexes with MMP-1, haptoglobulin, CD44, CD91, Fetuin A and proteoglycan.[17] The interaction with CD44 may be especially relevant to cell mobility.[18] The MMP-9 homodimer is formed through interactions between blade IV of the hemopexin-like domain whereas heterodimeric interactions tend to be mediated by blade 1.[19] Peptides mimicking these regions of MMP-9 suppress cancer cell mobility suggesting that the oligomeric forms of MMP-9 may be useful to target in preventing cancer cell invasion.[20] Selectivity between the MMP-9 homodimer form and monomer form could be anticipated if an inhibitory dimer could occupy two adjacent MMP-9 sites thereby increasing affinity relative to monomeric forms. The inhibitor groups would need to be spaced at the correct distance and; therefore, inhibitor potency/selectivity might emerge as a function of spacer length. For example, potency and selectivity of opioid agonist homodimers was found to be a function of spacer length, leading to new insights into the disposition of G-protein coupled receptors.[21] Homodimerization may also cause inhibitory groups to bind at exosites in a non-specific fashion that could impart favourable binding or selectivity properties. The effect of homodimerization has since been investigated in numerous areas of medicinal chemistry including HIV protease inhibitors, cholinesterase inhibitors, kinase inhibitors and MMP-inhibitors.[22–24] Recent studies have reported on the dimer approach to inhibition of MMP-1, -2, -9, and -14, based on the hydroxamic acid moiety.[25] These compounds exhibited useful potency but disappointing selectivity between MMPs. We also observed little selectivity of tested inhibitors on the set of MMPs suggesting that the homodimers were not suitably arranged to make connections with secondary active or exo-sites, which could impart selectivity.[6,26] However, purified recombinant enzymes may not be ideal for assessing homodimer properties and functional potential in this context because they cannot heterodimerize. Indeed, polyglycosylation, which is absent in recombinant human MMP-9, is required for its homo-dimerization.[17] Therefore, selected dimer compounds with various spacer lengths were evaluated in a functional assay in which MMP-9 homo- and heterdimerization is possible and where the homodimer is believed to play a functional role. The inhibitors were evaluated in a cancer cell invasion assay at 10 μM and/or 100 nM. Matrigel membranes with 8.0 μm pore were used, while hepatocyte growth factor (HGF) was used to stimulate growth and invasiveness of Caco-2 cells.[27] Compound **3** at 10 μM inhibited 70% Caco-2 cell invasion (Fig. 1). Even at 100 nM, compounds **2**, **3**, **4**, **5**, **8**, and **13** also significantly reduced Caco-2 cell invasion. However, there was no evidence of a relationship between linker size and inhibitory effect suggesting that the dimeric compounds acted at a single site to inhibit invasion.

Finally, in order to assess the suitability of the compounds to transit the gastrointestinal tract, **3** was incubated at 37 °C in simulated human duodenal fluid and the amount of compound remaining assessed by RP HPLC as a function of time. Greater than 50% of **3** remained at 6 h, the typical oro-caecal transit time.

It has proven extremely difficult to produce clinically useful compounds that target MMP active sites because of enzyme similarity and polyfunctionality. Simultaneous targeting of active site and exosites or secondary active sites in oligomeric MMPs is a promising means of improving selectivity and therapeutic index since it is more closely aligned with function.[6] Due to their mass, polyvalent inhibitors may also be confined within the intestinal system from the oral route, another promising means of targeting specific MMP subpopulations. The distribution, function and accessibility of intestinal MMPs will be important in determining efficacy in this case. The compounds described are suitable for investigating this question because of their size and stability. They are being assessed currently using animal models of IBD. Dimeric compounds have additional potential to selectively inhibit the functionally important dimeric form of MMP-9, provided that dual occupancy increases affinity over physiological forms of this and related enzymes. The present study will hopefully advance efforts in this area. Homo- and hetero-dimeric compounds with greater linker chain length and hydrophilicity need to be investigated.

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