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ScienceDirect
Bioorganic & Medicinal Chemistry 14 (2006) 7539–7550
Bioorganic & Medicinal Chemistry

Design, synthesis and molecular modeling study of iminodiacetyl monohydroxamic acid derivatives as MMP inhibitors

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Received 3 May 2006; revised 28 June 2006; accepted 3 July 2006
Available online 27 July 2006

Abstract—As the matrix metalloproteinases (MMPs) can be massively up-regulated in degenerative tissues and degrade the extracellular matrix, these key enzymes are promising targets for the therapy of cancer and other degenerative diseases. Here, we are presenting a series of new non-peptidic hydroxamate-based matrix metalloproteinase inhibitors, MMPIs, incorporating the iminodiacetic (IDA) hydroxamic acid scaffold, as mimics of truncated peptidic MMPIs. A series of alkylaryl and sulfonylaryl groups, on the IDA basic scaffold, was investigated with the aim of improving potency and selectivity against MMPs involved in degenerative diseases. The sulfonamide based IDA derivatives studied (compounds **B1**–**B3**) showed to be potent (nM range) against deep S1′ pocket MMPs enzymes (i.e., MMP-2).

1. Introduction

The matrix metalloproteinases (MMPs) are secreted by cells or are membrane-bound proteases that catalyse the turnover of extracellular matrix (ECM) components.[1,2] Abnormal MMP activities are linked to many serious diseases. Tissue inhibitors of metalloproteinases (TIMPs) are the endogenous inhibitors of MMPs but, in cancer or in other degenerative pathologies, their misregulation leads to an 'overexpression' of some of these proteases.[3] The human MMPs family includes at least 23 enzymes which, on the basis of their substrate specificity, can be divided into four sub-groups: collagenases (MMP-1, -8, and -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, and -11) and membrane-type MMPs (MMP-14, -15, -16, and -24).[4]

Due to the relevant therapeutic potential associated with the inhibition of these enzymes, MMPs have been a major target of drug-based medicinal chemistry research.[5,6] All these metalloproteinases use the Zn^{2+} ion in their active site to catalyse the hydrolytic cleavage of amide bond of their natural peptide substrates.[7,8] Thus, the metalloproteinase inhibitors (MMPI) contain a zinc-binding group (ZBG) linked to different scaffolds to ensure strong interactions within the co-factor-binding regions of these enzymes; among them, the most potent and widely studied class of MMPI utilizes a hydroxamate as the ZBG.[5,6]

Some MMPs play a significant role in the ECM regulating key functions of some types of cells in degenerated tissues, facilitating metastatic tumour dispersion and angiogenesis, resistance to apoptosis and activation of EGF receptors.[9] In the past, some potent 'broad spectrum' synthetic MMPIs have been proposed and tested against tumours. Many of these new molecules have caused a severe musculoskeletal syndrome, with fibroproliferative effects in the joint capsule of the knee.[10–12] These effects are thought to be linked to an impairment of normal tissue remodelling governed by MMP-1 and/or by sheddases such as TNF-α-convertase.[13] For these reasons, a lack of activity with respect to MMP-1 is considered to be an important factor for the reduction of some side effects found for 'non-selective' MMPIs.[14] Recent developments on synthetic MMPIs, possessing good potency and high selectivity against some MMPs over-expressed in degenerate

Keywords: Matrix metalloproteinases; Hydroxamate; MMP inhibitors; Enzyme inhibitors.
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doi:10.1016/j.bmc.2006.07.011

tissues (MMP-2, -3, -8, -13, and -14) and showing very important cytostatic or cytotoxic effects, confirm the validity of their use as potential drugs in many pathologies. Nowadays new compounds, possessing selective inhibitory activity on MMPs, are in great demand and many research groups and pharmaceutical companies are involved in the improvement of new synthetic MMPIs, not only to develop new therapeutics but also to discover new agents to be used as diagnostics in cancer.[15,16]

In spite of disagreeing results obtained by hydroxamic acid MMPIs in clinical trials, probably due to their ability to tightly bind high transition-metal ions causing toxic side effects linked to the lack of metal binding selectivity,[5] we decided to continue our studies on this type of inhibitors, considering that some very recent hydroxamate-based highly selective MMPIs are actually in advanced trials.[6] We have recently reported preliminary developments of a new class of non-peptidic hydroxamate-based inhibitors, as derivatives of *N*-aryliminodiacetic acid (IDA).[17] We started with a readily made compound, *N*-((hydroxycarbamoyl)methyl)-*N*-benzylaminoacetic acid, **A0** in Chart 1, which mimics truncated potent peptidic succinyl-hydroxamate MMPIs, such as marimastat or batimastat.[18] In order to improve the potency and selectivity versus MMP-2 and MMP-13, two MMPs considered to be real targets in cancer therapy today, we planned a series of chemical/structural modifications on compound **A0** potentially able to yield new MMP-1/MMP-14-sparing hydroxamate inhibitors.[6] Since the specificity of the MMP inhibition is determined by the inhibitor's ability to interact inside the S1′ region of the enzyme active site, we investigated and report here the introduction of a series of alkylaryl and sulfonylaryl substituent groups at the IDA nitrogen (compounds **A** and **B**, see Chart 1) and the effect that their lipophilic, electrostatic and stereochemical characteristics could have on diverse inhibitor-enzyme binding interactions; furthermore the effect of replacing the carboxylic moiety of compound **A0** by an amide chain, containing an alkylaryl-piperazine group, is also evaluated (compound **C1**).

2. Chemistry

The synthetic routes for the preparation of the new compounds, here designated as **A**, **B**, **C**, are reported in Schemes 1 and 2. For the preparation of compounds **A** and **B** (Scheme 1), the first step involved the IDA N-coupling with the appropriate alkylaryl or sulfonylaryl halides (R^1X), to give the corresponding N-substituted IDA intermediates, **A1a**–**A6a**, **B1a**–**B3a** (in this

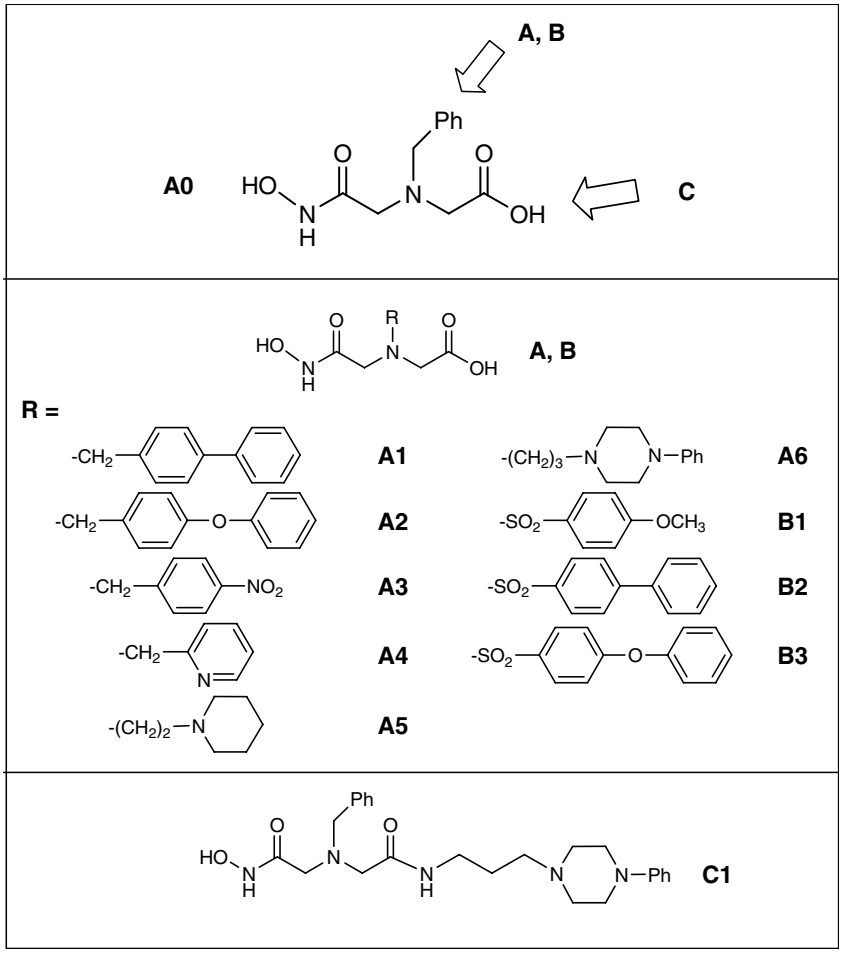
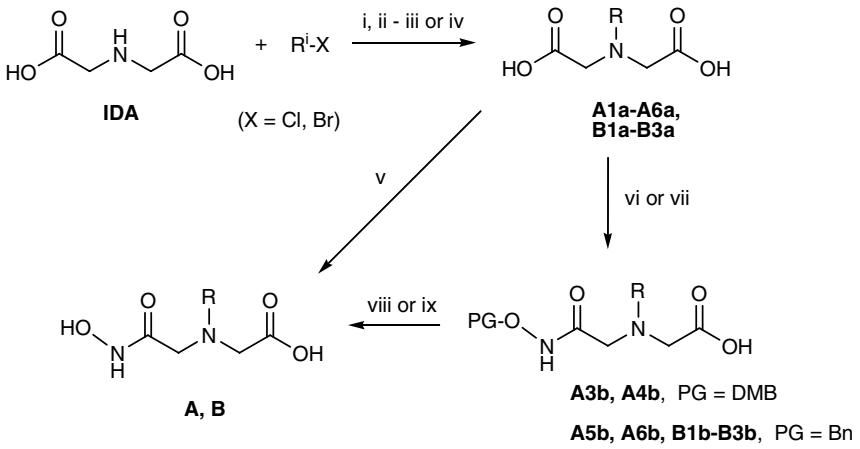
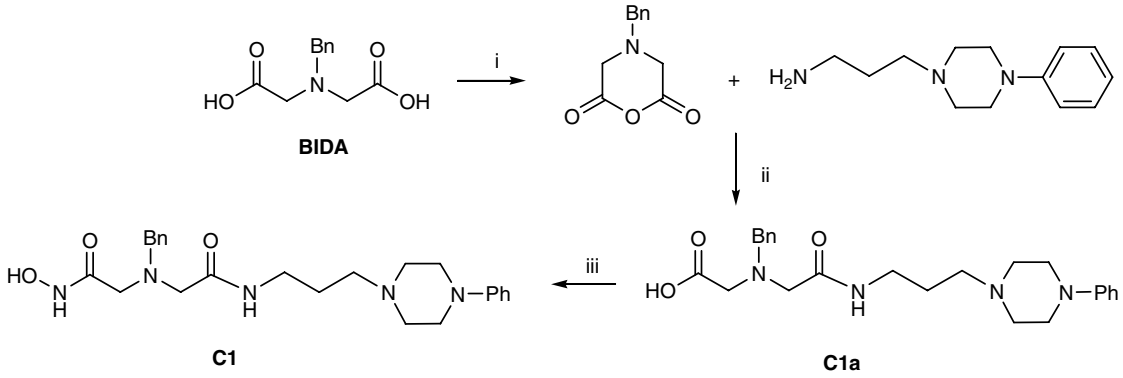


Chart 1.



Scheme 1. Synthesis of compounds **A** and **B**. Reagents and conditions: for **A1a**, **A2a**, **A4a**–**A6a**, (i) KOH, CH_3OH, reflux; for **A3a**: (ii) TEA, 3 equiv RCl, CH_3CN, reflux; (iii) 1:1 THF/2 M NaOH, rt; for **B1a**–**B3a**, (iv) KOH, 1:5 H_2O/THF; for **A1**, **A2**, (v) (ECF, NMM)/THF, NH_2OH/CH_3OH, 0 °C; for **A3b**, **A4b**, (vi) (ECF, NMM)/THF, NH_2ODMB/CH_3OH, 0 °C; for **A5b**, **A6b**, **B1b**–**B3b**, (vii) (ECF, NMM)/THF, NH_2OBn/MeOH, 0 °C; for **A3**, **A4**, (viii) TFA, CH_2Cl_2, rt; for **A5**, **A6**, **B1**–**B3**, (ix) H_2, Pd/C, MeOH, rt.



Scheme 2. Synthesis of compound **C1**. Reagents and conditions: (i) $C_2O_2Cl_2$, CH_2Cl_2, reflux; (ii) TEA, CH_3CN, 0 °C; (iii) (ECF, NMM)/THF, NH_2OH/CH_3OH, 0 °C.

compound designation, the suffix '**a**' refers to the respective di-carboxylic acid intermediate). Depending on the intermediate type, different reaction conditions have been used, namely, a homogeneous phase for compounds **A1a**–**A6a** and Schotten–Baumann conditions for compounds **B1a**–**B3a**.

The second step consisted of the condensation of one carboxylic group of the N-substituted IDA with hydroxylamine, upon previous activation of that group with ethylchloroformate (ECF) in the presence of *N*-methylmorpholine (NMM). For the preparation of some compounds, the hydroxylamine was *O*-alkyl protected (alkyl = benzyl for compounds **A3b**–**A6b**, **B1b**–**B3b**, and alkyl = 2,4-dimethoxybenzyl, DMB, for compounds **A3b** and **A4b**); here, the suffix '**b**' refers to the respective intermediates containing a carboxylic acid and an O-protected hydroxamic acid, whenever it exists. The DMB O-protection was selected to avoid the undesirable removal of *N*-alkyl substituents by catalytic hydrogenolysis with palladium, the usual method for *O*-benzyl deprotection. The *O*-DMB protected hydroxylamine was prepared by amination of the dimethoxybenzyl alcohol, according to the literature.[19,20] For these intermediates, the DMB removal was otherwise carried out in acidic media using a 5% trifluoroacetic acid dichloromethane solution.

Compound **C1** was prepared by first monoamidation of the *N*-benzyliminodiacetic anhydride with 3-(4-phenyl-piperazine-1-yl)-propylamine, (Scheme 2), followed by condensation of the second carboxylic group with hydroxylamine, in the presence of ECF and NMM;3-(4-phenyl-piperazine-1-yl)-propylamine was prepared by standard methods.[21]

3. MMPs inhibition

The hydroxamic acid derivatives of the *N*-alkyl- and *N*-alkylaryl-IDAs, compounds **A** and **C**, and some of their *N*-arylsulfonamide analogues, compounds **B**, were firstly tested on a limited panel of MMPs (MMP-2, -7, -9, and -14).[22] For the most active compounds

(compounds **B**), tests were expanded to a larger set of MMPs (MMP-1, -3, -8, -13, and -16), to obtain a complete inhibitory profile. Table 1 reports MMP inhibitory activities of these compounds as IC_{50} values, as well as the corresponding results obtained for CGS 27023A, as a reference inhibitor.

All *N*-alkylaryl-IDA hydroxamic acid derivatives were devoid of significant inhibitory activity on the MMPs screened, showing IC_{50} values in the micromolar range. Among these IDA derivatives, only the *N*-4-phenylbenzyl derivative (**A1**) showed IC_{50} values below 100 μM for some of the selected MMPs (79.4 μM for MMP-2 and 70.8 μM for MMP-9).

However, their sulfonamide analogues (compounds **B**) showed high inhibitory potency against the MMPs screened, with IC_{50} values in the nanomolar range, thus a comparable or improved potency in comparison with the reference drug (CGS 27023A). The *p*-methoxybenzene-sulfonamide (**B1**) showed moderate/good inhibitory potency against MMP-2, -7, -9, and -13, with IC_{50} values in the range of 0.2–0.3 μM. The introduction of bulky/'lengthy' aromatic groups on the sulfonamide (P1′ group), namely on the *para* position of the benzenesulfonamide moiety, such as the rigid biphenyl sulfonamide (B2) or the more flexible and adaptable *p*-phenoxybenzene-sulfonamide (B3), showed a considerable improvement in their inhibitory potency against the MMPs characterized by a deep S1′ pocket, such as MMP-2, -8, -9, -13, -14, and -16. These results were in agreement with the previous SAR results on other classes of MMPI analogues. Among the MMPIs studied, the most potent inhibitor was the *p*-phenoxybenzyl sulfonamide derivative, **B3**, with IC_{50} ranging from 1 to 30 nM, according to the following order MMP-2 > MMP-13 > MMP-9 > MMP-8 > MMP-16 > MMP-14. Furthermore the *N*-arylsulfonamide-based inhibitors showed also very low activity towards MMP-1, an anti-target MMP whose inhibition determined musculoskeletal side effects in old classes of MMPIs.[23]

4. Molecular modelling

In order to further understand and characterize the interaction of these inhibitors with MMP active sites, the two most promising ligands (**B2** and **B3**) were analyzed by docking modelling studies into a MMP-2 model.

To date, no X-ray structures of MMP-2-inhibitor complexes have been reported. Therefore, the crystallographic structure of the MMP-2 catalytic domain (1QIB[24]) was complexed with a known MMP-2-inhibitor ((*R*)-*N*-hydroxy-2-(*N*-isopropoxybiphenyl-4-ylsulfonamido)-3-methylbutanamide[25]). The model complex was minimized (see Section 6 for further details) and the resulting MMP-2 structure was used as a starting point for the docking studies.

Two inhibitors were docked into that MMP-2 model using the AUTODOCK 3.0 program[26] and the first

Table 1. Inhibitory activity of compounds **A**, **B**, and **C** compared with the reference drug CGS27023A towards the selected MMPs

Compound	IC_{50} (nM)								
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-13	MMP-14	MMP-16
A0		$>300\times10^3$		$>300\times10^3$		$>300\times10^3$		$>300\times10^3$	
A1		$(79.4\pm6.6)\times10^3$		$(161.3\pm6.9)\times10^3$		$(70.8\pm0.87)\times10^3$		$(116.3\pm4.9)\times10^3$	
A2		$(238\pm18)\times10^3$		$(277\pm7.4)\times10^3$		$(74.6\pm0.93)\times10^3$		$(138\pm3.5)\times10^3$	
A3		$(178\pm6)\times10^3$		$(300\pm14)\times10^3$		$>300\times10^3$		$(313\pm25)\times10^3$	
A4		n.a.		n.a.		$>300\times10^3$		$(200\pm4.4)\times10^3$	
A5		$(217.4\pm7.4)\times10^3$		n.a.		$(300\pm6.3)\times10^3$		$(294.1\pm8.9)\times10^3$	
A6		$(234.4\pm4.6)\times10^3$		n.a.		$>300\times10^3$		$(172.4\pm6.7)\times10^3$	
B1	$(6.77\pm0.58)\times10^3$	193 ± 20	$(1.38\pm0.13)\times10^3$	320 ± 62	1014 ± 43	322 ± 32	213 ± 7	$(1.69\pm0.15)\times10^3$	996 ± 24
B2	$(2.58\pm0.30)\times10^3$	46.1 ± 2.4	$(1.38\pm0.16)\times10^3$	$(23.8\pm1.6)\times10^3$	85.0 ± 7.0	155 ± 5	27.1 ± 2.6	1421 ± 84	348 ± 18
B3	$(1.53\pm0.06)\times10^3$	1.2 ± 0.1	37.2 ± 0.2	$(4.7\pm0.2)\times10^3$	3.7 ± 0.2	3.2 ± 0.2	1.6 ± 0.1	27.9 ± 1.7	11.4 ± 0.6
C1		n.a.		n.a.		$>300\times10^3$		$>300\times10^3$	
CGS27023A	56.3 ± 5.8	24.8 ± 4.0	22.0 ± 1.4	100.0 ± 7.8	7.7 ± 1.2	5.0 ± 1.3	5.5 ± 1.1	23.2 ± 1.6	6.8 ± 0.8

n.a., means non-active compounds.

structure of the first cluster (see Section 6 for details) was used as starting geometry for the molecular dynamics (MD) calculations. One nanosecond of the MD run was carried out for the two complexes, as shown in Figure 1a; after 100 ps of the MD, both the complex systems reached equilibrium, since the total energy for the last 900 ps remained constant. Analyzing the root mean square deviation (RMSD) from the X-ray structure of all the heavy atoms of the complexes, we observed that after an initial increase, the RMSD did not exceed the value of 1.2 Å (see Fig. 1b), suggesting that our MD procedure was correct.

Figure 2 shows that the biphenyl group of compound **B2** is inserted into the S1′ pocket and the hydroxamate group interact with the Zinc metal ion and the carboxylate substituent is directed towards the S2′ region. Moreover, the hydroxamate group forms two H bonds with Ala165 and Glu202 (1QIB sequence number), while the oxygen atoms of the sulfonamido group interacts with Leu164 and Ala165.

Compound **B3** is about 50-fold more potent than **B2** although it only differs by the presence of a *p*-phenoxybenzyl instead of the biphenyl group on their P1′ position. Our docking studies suggest that **B3** has an overall binding geometry very similar to that of **B2**, with all the H bonds preserved.

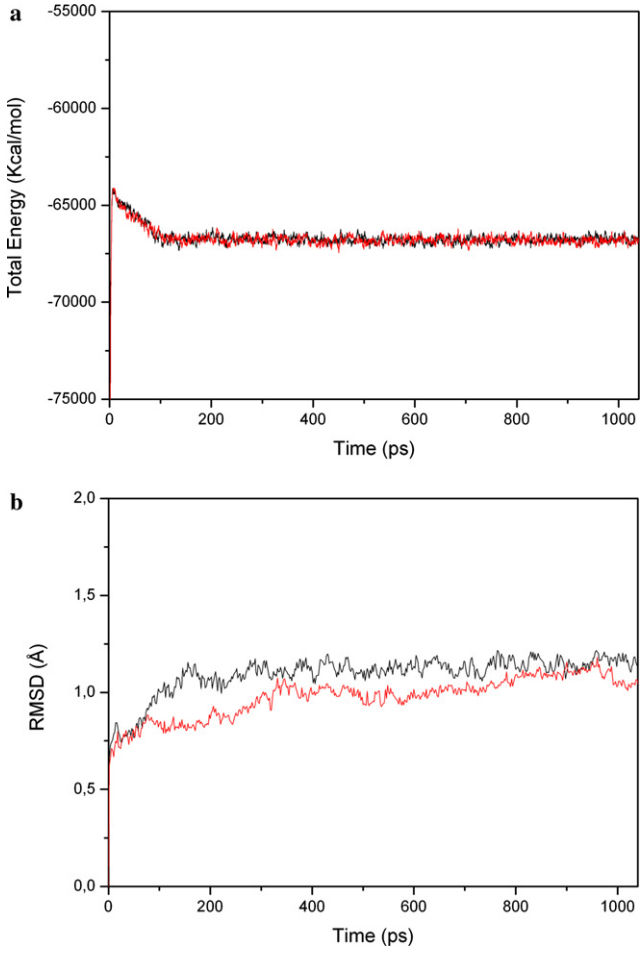


Figure 1. Results of the MD simulation of compounds **B2** (black) and **B3** (red) complexed with MMP-2. (a) Total energy (kcal/mol) of the system plotted versus time (ps); (b) root-mean-square deviation (RMSD) in angstroms (Å), between the protein and the starting X-ray structure for all the heavy atoms, versus time (ps).

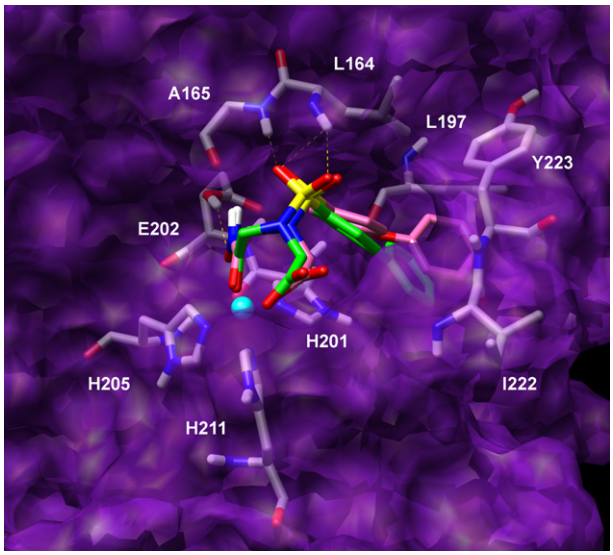


Figure 2. Superimposition of compounds **B2** and **B3** (respectively, coloured green and magenta) in the MMP-2 binding site.

The main differences between the two complexes concerned the interaction of the second aromatic ring in the S1′ pocket: the presence in **B3** of an oxygen atom between the two aromatic rings allowed a lipophilic interaction of the second phenyl ring with Leu197 and Tyr223 (3.5 and 4.8 Å, respectively). In the **B2**–MMP-2 complex these two interactions were weaker, since Leu197 and Tyr223 were, respectively, at 4.0 and 6.0 Å away from the biphenyl system. Furthermore, these two residues were also conserved in MMP-3, -8, -9, -13, -14, and -16 (in MMP-1, Leu197 is substituted with Arginine); thus the different spatial disposition of the biphenylether substituent in the S1′ pocket might contribute to explaining the best inhibitory activity of **B3** against this set of MMPs.

5. Conclusions

The results obtained for compounds with different P1′ substitutions emphasize the key role of the S1′ site to obtain potent and selective inhibitors. This has been found by others as well.[27] Lipophilic, electrostatic and steric characteristics are able to strongly modify MMPI potency and selectivity. In the IDA nitrogen-substituted inhibitors, the presence of the sulfonyl group (compounds **B1**–**B3)** is crucial, enabling its oxygen atoms to establish two fundamental H-bonds with the protein backbone (Leu164 and Ala165 for MMP-2), and also orientating the lipophilic groups in the S1′ pocket. Likewise, the absence of the sulfonyl group and its substitution with a methylenic alkyl chain lead to a considerable loss of MMPI activity not only due to the lack of two H bonds but also to the lack of a correct orientation of the extended aromatic portion of type **A** compounds in the

S1′ deep site. However the high activity and MMP-1/MMP-2 selectivity shown by compounds **B1**–**B3** encourage us to further explore the IDA scaffold to develop new more selective MMPIs.

6. Experimental

6.1. General methods

Analytical grade reagents were purchased from Aldrich, Sigma and Fluka and were used as supplied. Solvents were dried according to standard methods.[28] The chemical reactions were monitored by TLC using alumina plates coated with silica gel 60 F_{254} (Merck). Column flash chromatography separations were performed on silica gel Merck 230–400 mesh ASTM. Melting points were measured with a Leica Galen III hot stage apparatus and are uncorrected. IR spectra were recorded on a Bio-Rad Merlin, FTS 3000 MX. The ^{1}H NMR spectra were recorded on a Varian Unity 300 spectrometer at 25 °C. Chemical shifts are reported in ppm (δ) from standard internal references, namely, tetramethylsilane (TMS) for organic solvents and sodium 3-(trimethylsilyl)-[2,2,3,3-d4]-propionate (DSS) for D_2O solutions. The following abbreviations are used: s = singlet; d = duplet; t = triplet; q = quintuplet; m = multiplet; br s = broad singlet. Mass spectra (FAB) were recorded in a VG TRIO-2000 GC/MS instrument and ESI spectra on a Quattro LC mass spectrometer (Micromass, Manchester). The high resolution mass spectra (HRMS) were obtained with a high resolution Fourier transform ion cyclotron resonance (FTICR) instrument, Finnigan FT/MS 2001-DT, equipped with a 3.0-T superconducting magnet, by electron impact (EI), typically with 15-eV electron beam energies, 5-micro emission currents and 150 °C sample temperatures. Elemental analyses were performed on a Fisons EA1108 CHNF/O instrument.

6.2. Synthesis of the compounds A–B

6.2.1. *N*-(4-Biphenylmethyl)iminodiacetic acid (A1a). A solution of IDA (0.30 g, 2.25 mmol), KOH (0.39 g, 6.98 mmol) and 4-(chloromethyl)biphenyl (0.50 g, 2.48 mmol) in methanol (30 mL) was refluxed overnight. The reaction mixture was filtered and the solvent evaporated. The residue was dissolved in dry methanol and filtered to remove inorganics; evaporation of the solvent and recrystallization from ethanol/acetonitrile afforded the pure potassium salt of the compound as a white solid (0.32 g, 42% yield). Mp 281–285 °C; ^{1}H NMR (D_2O): δ 7.70–7.66 (2d, 4H, Ar***H***), 7.52–7.38 (m, 5H, Ar***H***), 3.95 (s, 2H, C***H***$_2$Biph), 3.31 (s, 4H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 376 (M+2K−H), 338 (M+K).

6.2.2. *N*-((Hydroxycarbamoyl)methyl)-*N*-(4-biphenylmethyl)aminoacetic acid (A1). To a solution of **A1a** (0.278 g, 0.82 mmol) in dry THF (25 mL) at 0 °C were added ECF (0.091 mL, 0.94 mmol) and dry NMM (0.104 mL, 0.94 mmol); the mixture was stirred for 40 min and subsequently filtered. Meanwhile, hydroxylamine hydrochloride (0.072 g, 1.03 mmol) was neutralized with KOH (0.058 g, 1.03 mmol) in dry methanol (15 mL), by stirring at 0 °C for 30 min and filtration for KCl removal. The first solution with the activated carboxylic acid was dropwise added to the second one, containing the free hydroxylamine; the reaction mixture was stirred at 0 °C for 3 h, then filtered and the solvent evaporated. The residue was recrystallized from methanol/acetonitrile, and the solid obtained was washed with 0.01 M HCl to achieve the pure product as a white solid (40% yield). Mp 153–155 °C; ^{1}H NMR (D_2O): δ 7.74–7.69 (2d, 4H, Ar***H***), 7.54–7.43 (m, 5H, Ar***H***), 3.77 (s, 2H, C***H***$_2$Biph), 3.17 (s, 2H, C***H***$_2$CONHOH), 3.15 (s, 2H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 315 (M+H). Elem. Anal. calcd. ($C_{17}H_{18}N_2O_4{\cdot}0.2H_2O{\cdot}5{\cdot}HCl$): C, 60.66; H, 5.65; N, 8.32. Found: C, 60.71; H, 5.65; N, 8.03%.

6.2.3. 1-(Chloromethyl)-4-phenoxybenzene. To an ice-cooled suspension of diphenyl ether (1 g, 5.88 mmol) and paraformaldehyde (0.097 g, 3.23 mmol) in acetone (10 mL), concd HCl (10 mL) was added and gaseous HCl was bubbled through the solution for 7 h, while stirring at 0 °C. The HCl stream was stopped and the solution was then stirred overnight at rt. The solvent was evaporated and the residue put into water (30 mL), which was extracted with ethyl ether (3× 30 mL); the total organic phase was washed with water until neutral pH (2× 30 mL) and dried over anhydrous Na_2SO_4. After solvent evaporation, flash column chromatography was performed with 2:1 CH_2Cl_2/n-hexane, giving a colourless oil as the pure product (0.39 g, 30% yield). ^{1}H NMR (CD_3OD): δ 7.30–7.24 (2d, 4H, Ar***H***), 7.03 (t, *J* = 7.4 Hz, 1H, *p*-Ph***H***), 6.90 (d, *J* = 8.1 Hz, 2H, Ar***H***), 6.85 (d, *J* = 8.1 Hz, 2H, Ar***H***), 4.53 (s, 2H, C***H***$_2$Cl).

6.2.4. *N*-(4-Phenoxybenzyl)iminodiacetic acid (A2a). A procedure similar to **A1a** was followed with 1-(4-(chloromethyl)phenoxy)benzene, giving the pure product as a white hygroscopic solid (85% yield). Mp 189–191 °C; ^{1}H NMR (D_2O): δ 7.44–7.36 (2d, 4H, Ar***H***), 7.19 (t, *J* = 7.4 Hz, 1H, *p*-Ph***H***), 7.08 (d, *J* = 8.4 Hz, 2H, Ar***H***), 7.04 (d, *J* = 8.4 Hz, 2H, Ar***H***), 3.77 (s, 2H, C***H***$_2$PhOPh), 3.18 (s, 4H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 316 (M+H), 354 (M+K).

6.2.5. *N*-((Hydroxycarbamoyl)methyl)-*N*-(4-phenoxybenzyl)aminoacetic acid (A2). A procedure similar to **A1** was followed with **A2a**. A white solid was obtained as the pure product (30% yield). Mp 160–161 °C; ^{1}H NMR (D_2O): δ 7.43–7.36 (2d, 4H, Ar***H***), 7.19 (t, *J* = 7.2 Hz, 1H, *p*-Ph***H***), 7.09–7.02 (2d, 4H, Ar***H***), 3.69 (s, 2H, C***H***$_2$PhOPh), 3.13 (s, 2H, C***H***$_2$CONHOH), 3.11 (s, 2H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 331 (M+H). Elem. Anal. calcd ($C_{17}H_{18}N_2O_5$): C, 61.81; H, 5.49; N, 8.48. Found: C, 61.85; H, 5.32; N, 8.32%.

6.2.6. *N*-(4-Nitrobenzyl)iminodiacetic acid (A3a). To a suspension of IDA (1 g, 7.51 mmol) and TEA (3.44 mL, 24.8 mmol) in dry acetonitrile (60 mL), 4-nitrobenzyl chloride (4.25 g, 24.8 mmol) was added and the reaction mixture was left refluxing overnight. After cooling down, the mixture was filtered, the solvent evaporated, the residue was put into ethyl acetate

(100 mL) and extracted with 5% NaOH (100 mL). The organic phase was then washed with water (2× 50 mL), dried over anhydrous Na_2SO_4 and evaporated. The residue was dissolved in 1:1 THF/2 M NaOH (100 mL) and the solution was stirred overnight. The solvent was evaporated, the residue dissolved in water (100 mL) and then washed with CH_2Cl_2 (100 mL). The aqueous phase was evaporated and the residue recrystallized from methanol/acetonitrile, giving the pure di-sodium salt of the product as a pale solid (2.20 g, 94% yield). Mp > 350 °C; ^{1}H NMR (D_2O): δ 8.34 (d, J = 7.8 Hz, 2H, Ar***H***), 7.78 (d, J = 7.8 Hz, 2H, Ar***H***), 4.61 (s, 2H, C***H***$_2$PhNO$_2$), 3.85 (s, 4H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 313 (M−H+2Na).

6.2.7. *O*-(2,4-Dimethoxybenzyl)-hydroxylamine (NH_2ODMB). To a suspension of 2,4-dimethoxybenzyl alcohol (5.05 g, 30.0 mmol), hydroxyphthalimide (6.36 g, 39.0 mmol) and triphenylphosphine (10.23 g, 39.0 mmol) in dry dichloromethane (150 mL), a solution of 40% toluenic diethyl azodicarboxylate (DEAD) (17.9 mL, 39.0 mmol) in dry dichloromethane (10 mL) was slowly added and stirred vigorously under nitrogen overnight. The mixture was filtered and the liquid subsequently washed with 5% NaOH (2× 100 mL), water (2× 100 mL) and brine (100 mL); after evaporation of the solvent, a yellow solid was obtained. This solid (17.62 g) was dissolved in ethanol (200 mL), hydrazine hydrate (27.5 mL, 0.56 mol) was added and this solution was refluxed for 3 h. The solvent was evaporated and the solid residue was extracted with ethyl ether (4× 50 mL); the organic phase was extracted with cooled 2 M HCl (4× 50 mL), which was subsequently washed with dichloromethane (2× 50 mL). After raising the pH up to 10 with 7 M NaOH, the aqueous phase was extracted with dichloromethane (5× 50 mL), the organic phase washed with water (2× 100 mL) and dried over anhydrous Na_2SO_4. After the solvent evaporation, the pure product was obtained as a pale yellow oil (1.60 g, 29%). ^{1}H NMR (D_2O, pD ca. 3): δ 7.26 (d, J = 8.4 Hz, 1H, 6-Ph***H***), 6.61–6.57 (m, 2H, 3,5-Ph***H***), 4.75 (s, 2H, C***H***$_2$), 3.81 (s, 6H, OC***H***$_3$).

6.2.8. *N*-((2,4-Dimethoxybenzyloxycarbamoyl)methyl)-*N*-(4-nitrobenzyl)aminoacetic acid (A3b). To an ice-cooled suspension of **A3a** (0.5 g, 1.60 mmol) in dry acetonitrile (30 mL) were added ECF (0.16 mL, 1.68 mmol) and NMM (0.19 mL, 1.68 mmol); the reaction mixture was stirred for 40 min and then filtered. A solution of NH_2ODMB (0.34 g, 1.84 mmol) in dry acetonitrile (20 mL) was dropwise added to the previous solution at 0 °C, and that mixture was left stirring for 2 h. After solvent evaporation, the residue was put into 0.1 M NaOH (60 mL) and subsequently washed with CH_2Cl_2 (3× 40 mL); the aqueous solution was acidified (pH ca. 2–3) with 2 M HCl, extracted with CH_2Cl_2 (3× 60 mL) and the organic phase was dried over Na_2SO_4. Evaporation of the solvent gave the pure product as a pale solid (0.262 g, 38% yield). Mp 64–67 °C; ^{1}H NMR (D_2O): δ 8.10 (d, J = 8.1 Hz, 2H, (PhNO$_2$)***H***), 7.44 (d, J = 8.7 Hz, 2H, (PhNO$_2$)***H***), 7.28 (d, J = 8.1 Hz, 1H, 6-DMB***H***), 6.52 (s, 1H, 3-DMB***H***), 6.46 (d, J = 9.3 Hz, 1H, 5-DMB***H***), 4.74 (s, 2H, C***H***$_2$DMB), 3.80 (s, 3H, OC***H***$_3$), 3.67 (s, 3H, OC***H***$_3$), 3.55 (s, 2H, C***H***$_2$PhNO$_2$), 2.99 (s, 2H, C***H***$_2$CONH), 2.91 (s, 2H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 434 (M+H).

6.2.9. *N*-((Hydroxycarbamoyl)methyl)-*N*-(4-nitrobenzyl)-aminoacetic acid (A3). To a solution of **A3b** (0.084 g, 0.19 mmol) in CH_2Cl_2 (3.8 mL) was added trifluoracetic acid, TFA, (0.2 mL) and the mixture was stirred at rt for 20 min. After evaporation of the solvent, methanol was added and the insoluble solid was filtered off. Evaporation of the solvent and recrystallization of the residue with dry ethyl ether gave the pure final product as a pale brown solid (0.032 g, 58% yield). Mp 141–143 °C; ^{1}H NMR (D_2O): δ 8.32 (d, J = 6.9 Hz, 2H, Ar***H***) 7.76 (d, J = 8.1 Hz, 2H, Ar***H***), 4.52 (s, 2H, C***H***$_2$PhNO$_2$), 3.95 (s, 2H, C***H***$_2$CONHOH), 3.80 (s, 2H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 284 (M+H). Elem. Anal. calcd ($C_{11}H_{13}N_3O_6$): C, 46.65; H, 4.63; N, 14.84%. Found: C, 46.74; H, 4.60; N, 14.67%.

6.2.10. *N*-((Pyridin-2-yl)methyl)iminodiacetic acid (A4a). A solution of 2-picolyl chloride hydrochloride (1.36 g, 8.26 mmol) in methanol (40 mL) was neutralized with KOH (0.46 g, 8.26 mmol), left stirring for 30 min and the solid was filtered off. That solution was slowly added to a solution of IDA (1 g, 7.51 mmol) and KOH (1.31 g, 23.28 mmol) in methanol (75 mL) at 50 °C and was refluxed overnight. After filtration and evaporation of the solvent, the residue was dissolved in water (20 mL); this solution was washed with CH_2Cl_2 (4× 20 mL) and, after neutralization with 1 M HCl, it was evaporated and dried under vacuum. The solid residue was dissolved in dry methanol, filtered to remove inorganics and the solution was evaporated. Recrystallization of the residue with dry ethanol gave the pure potassium salt of the product as a white solid (1.39 g, 71% yield). Mp 103–106 °C; ^{1}H NMR (D_2O): δ 8.61 (d, J = 5.1 Hz, 1H, Py***H***), 7.93 (t, J = 7.5 Hz, 1H, Py***H***), 7.55 (d, J = 4.5 Hz, 1H, Py***H***), 7.50 (t, J = 6.0 Hz, 1H, Py***H***), 4.56 (s, 2H, C***H***$_2$Py), 3.83 (s, 4H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 263 (M+K).

6.2.11. *N*-((2,4-Dimethoxybenzyloxycarbamoyl)methyl)-*N*-((pyridin-2-yl)methyl)aminoacetic acid (A4b). A general procedure similar to that of **A3b** was followed, but the last extraction was performed at pH 5–6, giving a pale hygroscopic foam (54% yield). ^{1}H NMR (D_2O, pD ca. 9): δ 8.38 (d, 1H, J = 4.5 Hz, 1H, Py***H***), 7.76 (t, 1H, J = 7.8 Hz, 1H, Py***H***), 7.44 (d, 1H, J = 7.8 Hz, 1H, Py***H***), 7.31–7.26 (m, 2H, Py***H***, 6-Ph***H***), 6.51 (s, 1H, 3-Ph***H***), 6.44 (d, 1H, J = 8.4 Hz, 1H, 5-Ph***H***), 4.73 (s, 2H, C***H***$_2$Ph), 3.80 (s, 3H, OC***H***$_3$), 3.67 (s, 3H, OC***H***$_3$), 3.53 (s, 2H, C***H***$_2$Py), 2.98 (s, 2H, C***H***$_2$CONHOH), 2.92 (s, 2H, C***H***$_2$CO$_2$H); *m*/*z* (FAB): 390 (M+H), 412 (M+Na).

6.2.12. *N*-((Hydroxycarbamoyl)methyl)-*N*-((pyridin-2-yl)methyl)aminoacetic acid (A4). A procedure similar to **A3** was followed and a pale hygroscopic solid was obtained (50% yield). ^{1}H NMR (D_2O): δ 8.58 (d, J = 4.2 Hz, 1H, Py***H***), 8.10 (t, J = 7.6 Hz, 1H, Py***H***), 7.68 (d, J = 8.1 Hz, 1H, Py***H***), 7.59 (t, J = 6.3 Hz, 1H, Py***H***), 4.12 (s, 2H, C***H***$_2$Py), 3.48 (s, 2H, C***H***$_2$CON-

HOH), 3.38 (s, 2H, C***H***$_2$CO$_2$H); *m/z* (HRMS) Calcd for $(MNa)^+$: 262.0804. Found 262.0801.

6.2.13. *N*-(2-(Piperidin-1-yl)ethyl)iminodiacetic acid (A5a). A procedure similar to **A4a** was followed, but using 1-(2-chloroethyl)piperidine hydrochloride instead of 2-picolyl chloride hydrochloride. The crude product of the reaction was dissolved in dry methanol, filtered to remove the inorganic materials and then it was recrystallized from methanol/acetonitrile to give the pure potassium salt of the product as white hygroscopic solid (95% yield). ^{1}H NMR (D$_2$O, pD ca. 3): δ 3.80 (s, 4H, C***H***$_2$CO$_2$H), 3.53 (d, J = 11.7 Hz, 2H, NC***H***$_2$CH$_2$Pip), 3.36 (tt, 4H, Pip***H***), 2.97 (t, J = 11.8 Hz, 2H, NCH$_2$C***H***$_2$Pip), 1.95–1.70 (m, 5H, Pip***H***), 1.48 (t, J = 11.7 Hz, 1H, Pip***H***); *m/z* (FAB): 359 (M+2K+Cl+H).

6.2.14. *N*-((Benzyloxycarbamoyl)methyl)-*N*-(2-(piperidin-1-yl)ethyl)aminoacetic acid (A5b). To an ice-cooled suspension of **A5a** (1 g, 3.14 mmol) in dry THF (40 mL) were added ECF (0.30 mL, 3.14 mmol) and NMM (0.35 mL, 3.14 mmol), and the reaction mixture was stirred for 40 min, being filtered afterwards. *O*-Benzylhydroxylamine hydrochloride (0.552 g, 3.45 mmol) was neutralized with KOH (0.194 g, 3.45 mmol) in dry methanol (30 mL), stirred for 30 min and then filtered off the solids. The hydroxylamine solution was dropwise added to the first solution and the mixture was stirred at 0 °C for 2 h. The reaction mixture was filtered, evaporated, and the residue taken into water (25 mL) and washed with CH_2Cl_2 (5× 25 mL). The pH of the aqueous solution was raised up to 8 with 0.1 M KOH and further extractions were performed with CH_2Cl_2 (3× 25 mL). The aqueous phase was evaporated and the solid residue was extracted with dry methanol to remove the inorganics. Recrystallization from dry acetonitrile/ethyl ether yielded the pure product as a hygroscopic solid (0.273 g, 25% yield). ^{1}H NMR (D$_2$O, pD ca. 3): δ 7.42 (s, 5H, Ph***H***), 4.87 (s, 2H, C***H***$_2$Ph), 3.52 (s, 2H, C***H***$_2$CONHOBn), 3.47 (s, 2H, C***H***$_2$CO$_2$H), 3.35 (d, 2H, J = 11.7 Hz, 2H, NC***H***$_2$CH$_2$Pip), 3.08 (d, J = 10.8 Hz, 4H, Pip***H***), 2.86 (t, J = 11.0 Hz, 2H, NCH$_2$C***H***$_2$Pip), 1.89–1.67 (m, 5H, Pip***H***), 1.45 (t, J = 10.0 Hz, 1H, Pip***H***) *m/z* (FAB): 350 (M+H).

6.2.15. *N*-((Hydroxycarbamoyl)methyl)-*N*-(2-(piperidin-1-yl)ethyl)aminoacetic acid (A5). General procedure to remove the benzyl protecting group was followed: to a solution of **A5b** (0.200 g, 0.57 mmol) in methanol (10 mL) was added 10% Pd/C (0.050 g). The suspension was stirred at rt under H_2 (1.5 bar) for 4 h. After filtration and evaporation of the solvent, final recrystallization with CH_2Cl_2 yielded the pure product as a white hygroscopic solid (0.141 g, 95% yield). ^{1}H NMR (D$_2$O, pD ca. 3): δ 3.73 (s, 2H, C***H***$_2$CONHOH), 3.62 (s, 2H, C***H***$_2$CO$_2$H), 3.48 (d, J = 9.3 Hz, 2H, NC***H***$_2$CH$_2$Pip), 3.27 (br s, 4H, Pip***H***), 2.92 (t, J = 11.0 Hz, 2H, NCH$_2$C***H***$_2$Pip), 1.90–1.64 (m, 5H, Pip***H***), 1.44 (t, J = 8.5 Hz, 1H, Pip***H***); *m/z* (FAB): 260 (M+H). Elem. Anal. calcd ($C_{11}H_{21}N_3O_4 \cdot 0.3H_2OCH_3OH$): C, 48.57; H, 8.70; N, 14.16%. Found: C, 48.76; H, 8.69; N, 14.34%.

6.2.16. 1-(3-Bromopropyl)-4-phenylpiperazine. To a solution of 1,3-dibromopropane (3.73 mL, 36.9 mmol) in dry THF (50 mL) at 70 °C, another solution of phenylpiperazine (1.88 mL, 12.3 mmol) and triethylamime (1.70 mL, 12.3 mmol) in dry THF was dropwise added. The mixture was stirred for 4 h and cooled down. After filtration, the solvent was evaporated under vacuum, avoiding the temperature rising above 40 °C. The purification of the product was accomplished with flash column chromatography using 2:1 *n*-hexane/dichloromethane as an eluent, being the pure product obtained as a pale yellow hygroscopic solid (1.43 g, 41% yield). ^{1}H NMR ($CDCl_3$): δ 7.26 (t, J = 8.7 Hz, 2H, Ar***H***), 6.93 (d, 2H, J = 8.1 Hz, Ar***H***), 6.85 (t, 1H, J = 6.9 Hz, Ar***H***), 3.50 (t, 2H, J = 6.6 Hz, BrC***H***$_2$CH$_2$CH$_2$Pip), 3.20 (t, 4H, J = 5.0 Hz, 3,5-Pip***H***), 2.62 (t, 4H, J = 6.9 Hz, 2,6-Pip***H***), 2.55 (t, 2H, J = 7.5 Hz, BrCH$_2$CH$_2$C***H***$_2$Pip), 2.08 (q, 2HJ = 7.0 Hz, BrCH$_2$C***H***$_2$ CH$_2$Pip); *m/z* (FAB): 203 (M-Br).

6.2.17. *N*-(3-(4-Phenylpiperazin-1-yl)propyl)iminodiacetic acid (A6a). A procedure similar to that of **A4a** was followed using 1-(3-bromopropyl)-4-phenylpiperine. Final recrystallization from methanol yielded the pure product as a white hygroscopic solid (76% yield). ^{1}H NMR (D$_2$O): δ 7.41 (t, J = 7.6 Hz, 2H, Ar***H***), 7.16 (d, J = 8.1 Hz, 2H, Ar***H***), 7.08 (t, J = 7.0 Hz, 1H, Ar***H***), 3.62 (s, 4H, C***H***$_2$CO$_2$H), 3.31 (br s, 4H, 3,5-Pip***H***), 3.12 (t, 2H, J = 6.2 Hz, NC***H***$_2$CH$_2$CH$_2$Pip), 2.96 (br s, 4H, 2,6-Pip***H***), 2.86 (t, 2H, J = 6.2 Hz, NCH$_2$ CH$_2$C***H***$_2$Pip), 1.95 (q, 2H, J = 6.2 Hz, NCH$_2$C***H***$_2$ CH$_2$Pip); *m/z* (FAB): 374 (M+K), 336 (M+H).

6.2.18. *N*-((Benzyloxycarbamoyl)methyl)-*N*-(3-(4-phenylpiperazin-1-yl)propyl)aminoacetic acid (A6b). The reaction was similar to that for **A5b**. After evaporation of the reaction mixture, the residue was dissolved in CH_2Cl_2 (30 mL), extracted with 0.1 M KOH (4× 15 mL) and the aqueous phase was washed with ethyl acetate (2× 60 mL). After setting the pH to 5–6 with 2 M HCl, the aqueous phase was extracted with CH_2Cl_2 (10× 40 mL), the organic phases were dried over Na_2SO_4 and the solvent was evaporated. The residue was then recrystallized from THF, yielding the pure product as a white solid (0.34 g, 29% yield). Mp 134–135 °C; ^{1}H NMR (D$_2$O, pD ca. 8): δ 7.42–7.36 (m, 7H, Ar***H***), 7.13 (d, J = 8.1 Hz, 2H, Ar***H***), 7.06 (t, J = 7.1 Hz, 1H, Ar***H***), 3.16 (br s, 4H, 3,5-Pip***H***), 3.11 (s, 2H, C***H***$_2$CONH), 2.95 (s, 2H, C***H***$_2$CO$_2$H), 2.64 (br s, 4H, 2,6-Pip***H***), 2.37 (t, J = 8.1 Hz, 2H, NC***H***$_2$CH$_2$CH$_2$Pip), 2.28 (t, J = 7.8 Hz, 2H, NCH$_2$CH$_2$C***H***$_2$Pip), 1.61 (q, J = 6.5 Hz, 2H, NCH$_2$C***H***$_2$CH$_2$Pip); m/z (FAB): 441 (M+H).

6.2.19. *N*-((Hydroxycarbamoyl)methyl)-*N*-(3-(4-phenylpiperazin-1-yl)propyl)aminoacetic acid (A6). Benzyl deprotection of **A6b** was carried out according to the standard procedure used for **A5**. Recrystallization from dry acetonitrile gave the pure product as a white solid (86% yield). Mp 106–108 °C. ^{1}H NMR (D$_2$O, ca. pD 2): δ 7.45 (t, J = 7.6 Hz, 2H, Ar***H***), 7.33–7.25 (m, 3H, Ar***H***), 4.22 (s, 2H, C***H***$_2$CONHOH), 4.12 (s, 2H, C***H***$_2$CO$_2$H), 3.74

(br s, 4H, 3,5-Pip***H***), 3.65 (br s, 4H, 2,6-Pip***H***), 3.45 (t, $J = 7.6$ Hz, 2H, NC***H***$_2$CH$_2$CH$_2$Pip), 3.35 (t, $J = 8.0$ Hz, 2H, NCH$_2$CH$_2$C***H***$_2$Pip), 2.29 (q, $J = 5.0$ Hz, 2H, NCH$_2$C***H***$_2$CH$_2$Pip); *m/z* (FAB): 351 (M+H), 373 (M+Na); Elem. Anal. calcd ($C_{17}H_{26}N4O_4 \cdot 0.5H_2O$): C, 56.87; H, 7.57; N, 15.61%. Found: C, 57.12; H, 7.49; N, 14.93%.

6.2.20. *N*-(4-Methoxybenzenesulfonyl)iminodiacetic acid (B1a). To a solution of IDA (1 g, 7.51 mmol) and KOH (1.26 g, 22.53 mmol) in water (5 mL) was added a solution of 4-methoxybenzenesulfonyl chloride (1.86 g, 9.01 mmol) in THF (25 mL), and the mixture was stirred at rt for 1 day. After phase separation, the aqueous phase was washed with CH_2Cl_2 (2× 20 mL) and then evaporated. Recrystallization from methanol yielded the pure potassium salt of the product as a white solid (1.66 g, 65% yield). Mp 221–223 °C; ^{1}H NMR (D_2O): δ 7.80 (d, $J = 8.7$ Hz, 2H, Ar***H***), 7.12 (d, $J = 8.7$ Hz, 2H, Ar***H***), 3.99 (s, 4H, C***H***$_2$CO$_2$H), 3.88 (s, 3H, OCH$_3$); *m/z* (FAB): 342 (M+K).

6.2.21. *N*-((Benzyloxycarbamoyl)methyl)-*N*-(4-methoxybenzenesulfonyl)aminoacetic acid (B1b). A process similar to **A5b** was followed, but with **B1a**. The reaction mixture was filtered, the solvent evaporated, and the residue put into a 5% NaOH solution (50 mL) with filtration of the insoluble solid. The aqueous solution was washed with ethyl ether (2× 50 mL), acidified with 7 M HCl and subsequently extracted with ethyl ether (4× 50 mL). After drying the organic phase over anhydrous Na_2SO_4 and solvent evaporation, the residue was recrystallized from ethyl ether/petroleum ether to obtain the pure product as a white solid (0.230 g, 19% yield). Mp 125–127 °C; ^{1}H NMR (D_2O, pD ca. 9): δ 7.77 (d, $J = 7.8$ Hz, 2H, Ar***H***), 7.37 (s, 5H, Ph***H***), 7.06 (d, $J = 7.8$ Hz, 2H, Ar***H***), 4.72 (s, 2H, C***H***$_2$Ph), 3.87 (s, 3H, OC***H***$_3$), 3.73 (s, 2H, C***H***$_2$CONH), 3.68 (s, 2H, C***H***$_2$CO$_2$H); m/z (FAB): 309 (M+H).

6.2.22. *N*-((Hydroxycarbamoyl)methyl)-*N*-(4-methoxybenzenesulfonyl)aminoacetic acid (B1). Standard benzyl deprotection was performed with **B1b**, as described for **A5**. After filtration and solvent evaporation, the residue was recrystallized with CH_2Cl_2, yielding the pure product as a white solid (0.140 g, 90% yield). Mp 138–140 °C; ^{1}H NMR (D_2O): δ 7.83 (d, $J = 7.8$ Hz, 2H, Ar***H***), 7.15 (d, $J = 8.1$ Hz, 2H, Ar***H***), 4.09 (s, 2H, C***H***$_2$CONH), 3.98 (s, 2H, C***H***$_2$CO$_2$H), 3.90 (s, 3H, OC***H***$_3$); *m/z* (FAB): 319 (M+H). Elem. Anal. calcd ($C_{11}H_{14}N_2O_7$): C, 41.51; H, 4.43; N, 8.80; S, 10.07%. Found: C, 41.44; H, 4.41; N, 8.93; S, 10.24%.

6.2.23. *N*-(4-Biphenylsulfonyl)iminodiacetic acid (B2a). To a solution of IDA (0.40 g, 3.01 mmol) and KOH (0.524 g, 9.33 mmol) in water (5 mL), a solution of 4-biphenylsulfonyl chloride (0.837 g, 3.31 mmol) in THF (25 mL) was added, and the mixture stirred overnight at rt. The organic solvent was evaporated and after the addition of 5% NaOH (30 mL) the insoluble solid was filtered off. The aqueous solution was washed with CH_2Cl_2 (3× 30 mL) and, after acidification to pH 1–2 with 7 M HCl, extracted with ethyl acetate (3× 30 mL). The organic phase was dried over anhydrous Na_2SO_4 and, after solvent evaporation, the pure product was obtained as a white solid (0.643 g, 61% yield). Mp 166–169 °C; ^{1}H NMR (D_2O, pD ca. 8): δ 7.96 (d, $J = 7.8$ Hz, 2H, Ar***H***), 7.88 (d, $J = 8.1$ Hz, 2H, Ar***H***), 7.80 (d, $J = 7.2$ Hz, 2H, Ar***H***), 7.61–7.52 (m, 3H, Ar***H***), 4.00 (s, 4H, C***H***$_2$CO$_2$H); *m/z* (FAB): 388 (M+K), 350 (M+H).

6.2.24. *N*-((Benzyloxycarbamoyl)methyl)-*N*-(4-biphenylsulfonyl)aminoacetic acid (B2b). A procedure similar to **A5b** was followed starting from **B2a**. The pure product was obtained as a hygroscopic white solid (94% yield). ^{1}H NMR (D_2O, pD ca. 8): δ 7.91 (d, $J = 8.7$ Hz, 2H, Ar***H***), 7.81 (d. $J = 8.4$ Hz, 2H, Ar***H***), 7.74 (d, $J = 7.2$ Hz, 2H, Ar***H***), 7.55–7.50 (m, 3H, Ar***H***), 7.32 (s, 5H, CH$_2$Ph***H***), 4.66 (s, 2H, C***H***$_2$Ph), 3.85 (s, 2H, C***H***$_2$CONH), 3.76 (s, 2H, C***H***$_2$CO$_2$H); *m/z* (FAB): 455 (M+H).

6.2.25. *N*-((Hydroxycarbamoyl)methyl)-*N*-(4-biphenylsulfonyl)aminoacetic acid (B2). Catalytic hydrogenolysis of **B2b** was carried out as for **A5** but under 4 bar H_2. Final recrystallization from CH_3CN/ethyl ether yielded the pure product as a white solid (97% yield). Mp 148–150 °C; ^{1}H NMR (D_2O): δ 7.96 (d, $J = 8.4$ Hz, 2H, Ar***H***), 7.98 (d, $J = 8.4$ Hz, 2H, Ar***H***), 7.77 (d, $J = 7.2$ Hz, 2H, Ar***H***), 7.58–7.50 (m, 3H, Ar***H***), 3.87 (s, 2H, C***H***$_2$CONHOH), 3.85 (s, 2H, C***H***$_2$CO$_2$H); *m/z* (FAB): 365 (M+H). Elem. Anal: calcd ($C_{16}H_{16}N_2O_6S \cdot 0.7H_2O \cdot 0.9CH_3CN$): C, 51.57; H, 4.90; N; 9.75, S, 7.75%. Found: C, 51.75; H, 5.41; N, 9.51; S, 7.29%.

6.2.26. 4-Phenoxybenzenesulfonyl chloride. To an ice-cooled solution of diphenyl ether (0.93 mL, 5.88 mmol) in dry dichloromethane (30 mL), under N_2 atmosphere, was added chlorosulfonic acid (0.39 mL, 5.88 mmol), and the mixture stirred for 2 h at 0 °C. The solvent was then evaporated and the residue dried under vacuum, giving a pale pink hygroscopic solid (1.47 g, >99% yield); ^{1}H NMR ($CDCl_3$): 10.79 (s, 1H, SO$_3$***H***), 7.81 (d, $J = 9.0$ Hz, 2H, Ar***H***), 7.40 (t, $J = 8.4$ Hz, 2H, Ar***H***), 7.21 (t, $J = 7.5$ Hz, 1H, Ar***H***), 7.04 (d, $J = 8.4$ Hz, 2H, Ar***H***), 6.99 (d, $J = 8.4$ Hz, 2H, Ar***H***); *m/z* (FAB): 250 (M), 251 (M+H). A solution of the former solid (1.29 g, 5.15 mmol) and catalytic amount of DMF (tree drops) in thionyl chloride was refluxed for 6 h. After evaporation of the solvent, the residue was dissolved in ethyl ether (40 mL), the solution washed with 5% NaOH (2× 40 mL) and water (2× 40 mL) and then dried over anhydrous Na_2SO_4. After evaporation of the solvent, the pure product was obtained as a pinkish oil (1.28 g, 92% yield); ^{1}H NMR ($CDCl_3$): δ 7.98 (d, $J = 9.0$ Hz, 2H, Ar***H***), 7.46 (t, $J = 7.8$ Hz, 2H, Ar***H***), 7.28 (t, $J = 7.4$ Hz, 1H, Ar***H***), 7.12–7.07 (2d, 4H, Ar***H***); *m/z* (FAB): 233 (M-Cl), 268 (M).

6.2.27. *N*-(4-Phenoxybenzenesulfonyl)iminodiacetic acid (B3a). A procedure similar to that of **B2a** was followed using 4-phenoxybenzenesulfonyl chloride. A pale solid was obtained (51% yield). Mp 148–151 °C; ^{1}H NMR

(D_2O, pD ca. 8): δ 7.81 (d, J = 9.0 Hz, 2H, Ar***H***), 7.548 (t, J = 7.8 Hz, 2H, Ar***H***), 7.29 (t, J = 7.5 Hz, 1H, Ar***H***), 7.17 (d, J = 8.1 Hz, 2H, Ar***H***), 7.11 (d, J = 9.0 Hz, 2H, Ar***H***), 3.91 (s, 4H, C***H***$_2$CO$_2$H); *m/z* (FAB): 366 (M+H), 388 (M+Na).

6.2.28. *N*-((Benzyloxycarbamoyl)methyl)-*N*-(4-phenoxybenzenesulfonyl)aminoacetic acid (B3b). A procedure similar to that of **A5b** was followed with **B3a**. From recrystallization with ethyl ether/*n*-hexane, the pure product was obtained as a pale hygroscopic solid (73% yield); ^{1}H NMR (D_2O, pD ca. 8): δ 7.74 (d, J = 8.7 Hz, 2H, Ar***H***), 7.43 (t, J = 7.5 Hz, 2H, Ar***H***), 7.34–7.23 (m, 8H, Ar***H***), 7.10 (d, J = 8.1 Hz, 2H, Ar***H***), 7.02 (d, J = 8.4 Hz, 2H, Ar***H***), 4.68 (s, 2H, C***H***$_2$Ph), 3.73 (s, 2H, C***H***$_2$CONH), 3.66 (s, 2H, C***H***$_2$CO$_2$H); *m/z* (FAB): 471 (M+H).

6.2.29. *N*-((Hydroxycarbamoyl)methyl)-*N*-(4-phenoxybenzenesulfonyl)aminoacetic acid (B3). Following a procedure similar to that for **B2** but with **B3b**, the pure product was obtained as a white slightly hygroscopic solid (80%); ^{1}H NMR (D_2O, pD ca. 8): δ 7.85 (d, J = 8.7 Hz, 2H, Ar***H***), 7.49 (t, J = 7.2 Hz, 2H, Ar***H***), 7.30 (t, J = 7.2 Hz, 1H, Ar***H***), 7.20–7.15 (2d, 4H, Ar***H***), 3.83 (s, 2H, C***H***$_2$CONHOH), 3.81 (s, 2H, C***H***$_2$CO$_2$H); *m/z* (FAB): 381 (M+H). Elem. Anal. calcd ($C_{16}H_{16}N_2O_7S$): C, 50.52; H, 4.24; N; 7.36, S, 8.43%. Found: C, 50.55; H, 4.45; N, 7.22; S, 8.17%.

6.3. Synthesis of the compounds C

6.3.1. *N*-Benzyl-iminodiacetic anhydride. To a suspension of *N*-benzyliminodiacetic acid (BIDA) (0.500 g, 2.24 mmol) in dry dichloromethane (50 mL) was added oxalyl chloride (0.25 mL, 2.80 mmol) and the reaction mixture was left under reflux for 3 h. After cooling down, the solid was filtered and washed with dry dichloromethane to yield the pure hydrochloric salt of the product as a white solid (0.505 g, 93% yield). ^{1}H NMR (CD_3CN): δ 7.54–7.47 (m, 5H, Ph***H***), 4.26 (s, 2H, C***H***$_2$Ph), 3.98 (s, 4H, C***H***$_2$CO); IR (KBr, cm^{-1}): 1794, 1737 ($\nu_{C=O}$).

6.3.2. 3-(4-Phenyl-piperazine-1-yl)-propylamine. To a solution of *N*-(3-bromopropyl)phthalimide (3.30 g, 12.3 mmol) in dry acetonitrile (40 mL) were added phenylpiperazine (1.88 mL, 12.3 mmol) and TEA (1.88 mL, 13.5 mmol). The mixture was left reacting under reflux for 7 h. After cooling down, the yellow solid formed was filtered and washed with acetonitrile, yielding the pure *N*-(3-(4-phenyl-piperazine-1-yl)propyl)phthalimide (3.34 g, 78% yield). Mp 132–134 °C. ^{1}H NMR ($CDCl_3$): δ 7.84 (m, 2H, Ar***H***), 7.68 (m, 2H, Ar***H***), 7.23 (t, J = 8.1 Hz, 2H, Ar***H***), 6.84 (m, 3H, Ar***H***), 3.80 (t, J = 6.9 Hz, 2H, NC***H***$_2$CH$_2$CH$_2$Pip), 3.04 (t, J = 8.8 Hz, 4H, 3,5-Pip***H***), 2.54 (t, J = 8.2 Hz, 4H, 2,6-Pip***H***), 2.48 (t, J = 7.5 Hz, 2H, NCH$_2$CH$_2$C***H***$_2$Pip), 1.91 (q, J = 7.0 Hz, 2H, NCH$_2$C***H***$_2$CH$_2$Pip); *m/z* (FAB): 349 (M).

To a solution of the former product (3.23 g, 9.24 mmol) in 2:3 H_2O/THF (40 mL) was added a 40% aqueous solution of methylamine (12 mL, 0.14 mol) and the reaction mixture was stirred at 60 °C overnight. The solvent was evaporated, the residue put into a 5% NaOH solution (40 mL) which was extracted with ethyl acetate (4× 40 mL), and the organic phase was dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was acidified with an HCl saturated methanolic solution and recrystallized with ethyl ether, producing the pure di-hydrochloride salt of the product as a yellow hygroscopic solid (2.00 g, 74% yield). ^{1}H NMR (D_2O): δ 7.40 (t, J = 8.1 Hz, 2H, Ar***H***), 7.15–7.07 (m, 3H, Ar***H***), 3.52 (br s, 8H, Pip***H***), 3.33 (t, J = 8.4 Hz, 2H, NH$_2$C***H***$_2$CH$_2$CH$_2$Pip), 3.10 (t, J = 7.6 Hz, 2H, NH$_2$CH$_2$CH$_2$C***H***$_2$Pip), 2.18 (q, J = 7.5 Hz, 2H, NH$_2$CH$_2$C***H***$_2$CH$_2$Pip); *m/z* (FAB): 220 (M+H).

6.3.3. *N*-Benzyl-*N*-(3-(4-phenyl-piperazine-1-yl)propylacetamide)aminoacetic acid (C1a). 3-(4-Phenyl-piperazine-1-yl)-propylamine di-hydrochloride (1.01 g, 3.45 mmol) was neutralized by stirring with KOH (0.39 g, 6.90 mmol) in dry methanol (50 mL) for 30 min. The solid was filtered off, the solvent evaporated and the residue dissolved in dry methanol/acetonitrile 1:1 (50 mL). To a suspension of BIDA anhydride hydrochloride (1.00 g, 4.14 mmol) in dry acetonitrile (40 mL) was added TEA (0.57 mL, 4.14 mmol) and the mixture stirred for 30 min. After cooling this solution with an ice-water bath, the amine solution was dropwise added and the solution stirred for 4 h at 0 °C. The reaction mixture was filtered, evaporated, and dried under vacuum. The crude material was dissolved in water (40 mL) and washed with CH_2Cl_2 (4× 40 mL). The pH of the aqueous phase was raised up to 7–8 with 2 M NaOH and then extracted with CH_2Cl_2 (4× 40 mL). The organic phase was dried over anhydrous Na_2SO_4 and after evaporation of the solvent and vacuum drying, the pure product was obtained as a hygroscopic white foam (1.23 g, 84% yield). ^{1}H NMR (D_2O): δ 7.51 (s, 5H, CH$_2$Ph***H***), 7.41 (t, J = 8.1 Hz, 2H, Ar***H***), 7.14–7.09 (m, 3H, Ar***H***), 4.48 (s, 2H, C***H***$_2$Ph), 4.09 (s, 2H, C***H***$_2$CONH), 3.88 (s, 2H, C***H***$_2$CO$_2$H), 3.82–3.67 (m, 4H, NC***H***$_2$CH$_2$C***H***$_2$Pip), 3.23–3.13 (m, 8H, Pip***H***), 1.90 (q, J = 7.2 Hz, 2H, NCH$_2$C***H***$_2$CH$_2$Pip); *m/z* (FAB): 425 (M+H).

6.3.4. *N*-Benzyl-*N*-(3-(4-phenyl-piperazine-1-yl)propylacetamide)aminoacetohydroxamic acid (C1). A procedure similar to that for **A1** was followed with **C1a**. Here, the crude product from evaporation of the reaction mixture was dissolved in CH_2Cl_2 (50 mL), the solution washed with water (4× 30 mL), dried over anhydrous Na_2SO_4 and the solvent evaporated. To the residue, THF was added leading to the precipitation of the pure product as a white solid (0.410 g, 48% yield). Mp 126–127 °C; ^{1}H NMR (D_2O, pD ca. 3): δ 7.54 (s, 5H, CH$_2$Ph***H***), 7.46 (t, J = 7.8 Hz, 2H, Ar***H***), 7.23–7.18 (m, 3H, Ar***H***), 4.57 (s, 2H, C***H***$_2$Ph), 4.19 (s, 2H, C***H***$_2$CONHOH), 4.13 (s, 2H, C***H***$_2$CONHCH$_2$), 3.54 (br s, 8H, Pip***H***), 3.26–3.23 (m, 4H, NC***H***$_2$CH$_2$C***H***$_2$Pip), 1.95 (q, J = 6.9 Hz, 2H, NCH$_2$C***H***$_2$CH$_2$Pip); *m/z* (FAB): 440 (M+H). Elem. Anal. calcd ($C_{24}H_{33}N_5O_3$·0.8HCl·0.2THF): C, 61.65; H, 7.39; N, 14.50%. Found: C, 61.77; H, 7.22; N, 14.54%.

6.4. MMP inhibition assays

Recombinant human progelatinases A (pro-MMP-2) and B (pro-MMP-9) secreted by transfected mouse myeloma cells were prepared in our laboratory.[22] Matrilysin (pro-MMP-7, human recombinant), MembraneType1-MMP (pro-MMP-14, human recombinant) and CD-MMP-16 (MT3-MMP catalytic domain) were purchased from Calbiochem. Proenzymes were activated immediately prior to use with *p*-aminophenylmercuric acetate (APMA 2 mM for 1 h at 37 °C for pro-MMP-2, APMA 1 mM for 1 h at 37 °C for pro-MMP-7 and pro-MMP-9) and with trypsin 5 μg/mL for 10 min at 37 °C followed by Soybean trypsin inhibitor (SBTI) 50 μg/mL for pro-MMP-14. For assay measurements, the stock solutions (100 mM) of the inhibitors in DMSO were further diluted, at seven different concentrations (1 nM–300 μM) for each MMP, as required in the fluorimetric assay buffer (FAB): Tris 50 mM, pH 7.5, NaCl 150 mM, $CaCl_2$ 10 mM, Brij 35 0.05% and DMSO 1%. The activated enzyme (final concentration 2.8 nM for MMP-2, 2.36 nM for MMP-7, 2.7 nM for MMP-9, 2.15 nM for MMP-14 and 2.2 nM for CD-MMP-16) and the inhibitor solutions were incubated in the assay buffer for 4 h at 25 °C. After the addition of 100 μM solution of the fluorogenic substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) in DMSO (final concentration 2 μM), the hydrolysis was monitored every 1 s for 10 min, by recording the increase in fluorescence (λ_{ex} = 328 nm, λ_{em} = 398 nm) using a Molecular Device M-2 Gemini plate reader. The assays were performed in duplicate in a total volume of 200 μL per well in 96-well microtitre plates (Corning, black, NBS). Control wells lack the enzyme or inhibitor. The MMP activity was expressed in relative fluorescent units (RFU). The percentage of inhibition was calculated against control reactions without the inhibitor. IC_{50} was determined using the formula: $V_i/V_o = 1/(1 + [I]/IC_{50})$, where V_i is the initial velocity of substrate cleavage in the presence of the inhibitor at concentration [I] and V_o is the initial velocity in the absence of the inhibitor. Results were analyzed using SoftMax Pro software and GraFit software.[29]

6.5. Automated docking procedure

The two ligands were submitted to a conformational search of 1000 steps. The algorithm used was the Montecarlo method with MMFFs as the forcefield and a distance-dependent dielectric constant of 1.0. The ligands were then minimized using the conjugated gradient (CG) method until a convergence value of 0.05 kcal/Å mol, using the same forcefield and the dielectric constant used for the conformational search.

Automated docking was carried out by means of the AUTODOCK 3.0 program[26]; AUTODOCK TOOLS was used to identify the torsion angles in the ligands, add the solvent model and assign the Kollman partial atomic charges to the protein. As regards the catalytic Zinc atom, the parameters published by Hu et al. [30,31] were used, while the ligand charges were calculated using the AM1-BCC method, as implemented in the Antechamber suite of Amber 8.

The regions of interest used by AUTODOCK were defined by considering (*R*)-*N*-hydroxy-2-(*N*-isopropoxybiphenyl-4-ylsulfonamido)-3-methylbutanamides complexed into MMP-2 as the central group; in particular, a grid of 54, 50 and 52 points in the *x*, *y* and *z* directions was constructed centred on the centre of the mass of this inhibitor. A grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used for the energetic map calculations.

Using the Lamarckian Genetic Algorithm, the docked compounds were subjected to 100 runs of the AUTODOCK search, in which the default values of the other parameters were used. Cluster analysis was performed on the results using an RMS tolerance of 1.0 Å and the best docked structures were used as starting points for the following MD simulations.

6.6. MD simulations

All simulations were performed using AMBER 8.0.[32] MD simulations were carried out at 300 K using the parm94 forcefield implemented with the Zinc bonded parameters that have recently been reported by us.[25] Explicit solvent model TIP3P water was used and the complexes were solvated with a 15 Å water cap. Sodium ions were added as counterions to neutralize the system. Prior to MD simulations, two minimization stages were carried out; in the first stage, we kept the protein fixed with a constraint of 500 kcal/mol and we just minimized the positions of the water molecules; then in the second stage, we minimized the entire system applying a constraint of 20 kcal/mol on the α-carbon. The two minimization stages consisted of 5000 steps with a combined algorithm, namely the sequential use of steepest descent (SD) and conjugate gradient methods, for the first 1000 and the last 4000 steps, respectively. Molecular dynamics trajectories were run using the minimized structure as a starting input, and the particle mesh Ewald (PME) algorithm was used for dealing with long-range interactions.[33] The time step of the simulations was 2.0 fs with a cutoff of 12 Å for the non-bonded interaction and SHAKE was employed to keep all bonds involving hydrogen atoms rigid. Constant-volume was carried out for 40 ps, during which the temperature was raised from 0 to 300 K (using the Langevin dynamics method); then, under constant-pressure, a 1 ns MD run was carried out at 300 K. All α-carbons not within 15 Å of the catalytic zinc were blocked with a harmonic force constant of 20 kcal/mol Å. The final structure of the complexes was obtained as the average of the last 600 ps of MD minimized with the CG method until a convergence of 0.05 kcal/Å mol.

The MMP-2 crystallographic structure was complexed with (*R*)-*N*-hydroxy-2-(*N*-isopropoxybiphenyl-4-ylsulfonamido)-3-methylbutanamides, as previously published,[25] and the complex was subjected to a preliminary minimization, as described above, subsequently the system was minimized, blocking all the α-carbons with a harmonic force constant of 20 kcal/mol Å, using the CG method until a convergence of 0.1 kcal/Å mol.

Acknowledgments

The authors thank the Portuguese Foundation for Science and Technology (FCT) (project POCTI/35344/QUI/00 and the doctoral Grant SFRH/BD/10714/2002) and the COST-D21-001 project program for financial support, as well as the Ministero dell'Università e della Ricerca Scientifica, PRIN 2004, for a financial contribution. The authors also thank Prof. Gillian Murphy, Department of Oncology, University of Cambridge, Hills Road, Cambridge, CB2 2XY, United Kingdom, for enzyme preparation and Dr. Elisa Nuti, Dipartimento di Scienze Farmaceutiche, Università di Pisa, via Bonanno 6, 56126 Pisa, Italy, for suggestions to improve enzyme inhibition tests.

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