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Carbamoylphosphonates, a New Class of in Vivo Active Matrix Metalloproteinase Inhibitors. 1. Alkyl- and Cycloalkylcarbamoylphosphonic Acids[1]

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Matrix metalloproteinases (MMPs) are a family of over 20 zinc-dependent enzymes that hydrolyze connective tissue and are involved in a variety of diseases, which are associated with undesired tissue breakdown. This paper reports the synthesis, characterization, and biological evaluation of a novel class of MMP inhibitors based on the carbamoylphosphonic acid function. We report a series of 10 open chain *N*-alkylcarbamoylphosphonic acids (ranging from R = C_1 to C_6 groups), eight *N*-cycloalkylcarbamoylphosphonic acids (ranging from cyclopropyl to cyclooctyl rings), and four *N*,*N*-dialkylcarbamoylphosphonic acids. The compounds were evaluated in three in vitro models, which consisted of (a) the in vitro invasion across a reconstituted basement membrane, (b) determination of the IC_{50} values on recombinant MMP-1, MMP-2 MMP-3, MMP-8, and MMP-9 enzymes, and (c) an in vitro capillary formation model, which is a model of angiogenesis. Several of the compounds were also tested in an in vivo murine melanoma model. The following general conclusions have been reached: Most compounds show selectivity for MMP-2 over the other MMP subtypes examined. Cycloalkylcarbamoylphosphonic acids are more potent than comparable open-chain alkyl compounds. Optimal activity against MMP-2 among the cycloalkyl derivatives was shown by *N*-cyclopentylcarbamoylphosphonic acid (**3m**). *N*,*N*-Dialkylcarbamoylphosphonic acids that were examined showed weak or no activity. The compounds examined showed toxic effects neither in vitro nor in vivo in the concentrations used. Carbamoylphosphonic acids are water soluble at physiological pH and are stable indefinitely.

Introduction

Matrix metalloproteinases (MMPs) are a family of structurally related zinc enzymes that mediate the breakdown of connective tissue and are therefore targets for therapeutic inhibitors in inflammatory, malignant, and degenerative diseases associated with excessive enzymatic activity.[2] Such conditions include cancer,[3] tumor metastasis,[4] arthritis,[5] and cardiovascular[6] diseases, as well as wound healing.[7] The Zn^{2+} ion present at the active sites of these enzymes is crucial for enzymatic activity; therefore, virtually all attempts to develop inhibitors have been based on so-called zinc-binding-groups (ZBG). The overwhelming majority of inhibitors reported contain a hydroxamic acid function as ZBG, and many of them have been reported to possess high inhibitory potency in vitro. Other MMP inhibitors synthesized contain carboxylic,[2,8] phosphonic,[2,9] and phosphinic[9,10] acids and some other functions as ZBGs.

We have been studying the chemical and biological properties of the acylphosphonic acids[11] (oxophosphonic acids) for some time and examined them as potential biological chelators. A representative bisacylphosphonate has recently been shown by X-ray crystallography to chelate calcium in a five-membered ring structure,[12] similar to the mode of zinc binding by hydroxamic acid inhibitors as shown by X-ray crystallography.[13] We have recently shown[14] that the stability constants of zinc complexes of some carbamoylphosphonates are significantly higher than those of the corresponding calcium complexes. Therefore, it seemed reasonable to examine such compounds as potential MMP inhibitors. In this paper, we describe the first group of a new class of selective MMP inhibitors based on the carbamoylphosphonic function as a new zinc binding group.

Results

Chemistry. First, we screened oxophosphonates available in our laboratory in an in vitro invasion assay, using a reconstituted basement membrane preparation which HT1080 tumor cells have to cross in order to disseminate. The molecules we examined initially included various alkanoyl- and aroylphosphonic diacids ($RCOPO_3H_2$ and $ArCOPO_3H_2$), alkyl- and aryloxycarbonylphosphonic ($ROCOPO_3H_2$ and $ArOCOPO_3H_2$), and alkylthiolcarbonylphosphonic acids ($RSCOPO_3H_2$), all of which showed significant but modest inhibitory potency (data not shown). In comparison, an analogous benzylphosphonic ($ArCH_2PO_3H_2$) acid and an aroylphosphonic acid monoester ($RCOPO_3HR'$) showed no activity at all in this assay. The breakthrough appeared when

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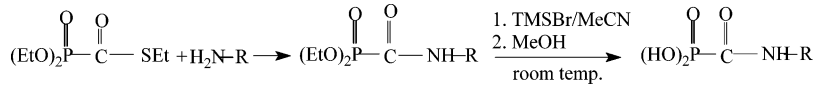
Scheme 1[a]



1 **2a -v** **3a - v**

[a] For the structures of R in compounds **a**−**v**, see Table 1.

Table 1. List of Compounds Reported in this Paper and the Results of Their in Vitro and in Vivo Evaluations

N-Alkylcarbamoylphosphonic Acids

	3	structure of R in R-NHCOPO$_3$H$_2$ (**3**)	invasion assay % inhibition at 50 μM[a]	recomb. MMP-1 IC$_{50}$ μM[b]	recomb. MMP-2 IC$_{50}$ μM[b]	recomb. MMP-3 IC$_{50}$ μM[b]	recomb. MMP-8 IC$_{50}$ μM[b]	recomb. MMP-9 IC$_{50}$ μM[b]	% inhibition of capillary formation at 50 μM[a]	% inhibition of lung metastasis formation[a,c]
1	**a**	methyl	40	>100	0.02	90	20	>100	-	55
2	**b**	ethyl	19	50	5.0	100	30	3.0	-	-
3	**c**	1-propyl	40	20	0.3	50	>100	20	-	-
4	**d**	1-butyl Na$^+$[d]	20	25	2	>100	>100	>100	20	-
5	**e**	Me$_2$CHCH$_2$[e]	inactive	40	>100	>100	1	>100	-	-
6	**f**	Me$_2$CH(CH$_2$)$_2$[e]	inactive	>100	30	>100	50	>100	-	-
7	**g**	PhCH$_2$CH$_2$	inactive	80	20	90	>100	>100	-	-
8	**h**	2-propyl	43	50	1.0	>100	50	2	55	40
9	**i**	(*RS*)-2-butyl	25	40	3	80	>100	>100	20	-
10	**j**	3-pentyl	46	80	3	>100	10	>100	-	50

N-Cycloalkylcarbamoylphosphonic Acids

	3	structure of R in R-NHCOPO$_3$H$_2$ (**3**)	invasion assay % inhibition at 50 μM	recomb. MMP-1 IC$_{50}$ μM	recomb. MMP-2 IC$_{50}$ μM	recomb. MMP-3 IC$_{50}$ μM	recomb. MMP-8 IC$_{50}$ μM	recomb. MMP-9 IC$_{50}$ μM	% inhibition of capillary formation at 50 μM	% inhibition of lung metastasis formation[c]
11	**k**	cyclopropyl	35	>100	0.5	>100	0.2	>100	-	-
12	**l**	cyclobutyl	53	>100	0.15	>100	0.04	>100	-	60
13	**m**	cyclopentyl	65	0.5	0.080	>100	>100	>100	70	72
14	**n**	cyclohexyl	46	0.1	3	3	>100	>100	50	37
15	**o**	cyclohexyl Et Li$^+$[f]	10	1.5	30	>100	>100	>100	-	-
16	**p**	cyclohexCH$_2$	50	30	0.2	1	80	1	53	69
17	**q**	cycloheptyl	45	>100	4	>100	>100	>100	45	-
18	**r**	cyclooctyl	33	>100	1	>100	4	>100	25	-

N,*N*-Dialkylcarbamoylphosphonic Acids

		structure of R$_2$N in R$_2$NCOPO$_3$H$_2$	invasion assay % inhibition at 50 μM	recomb. MMP-1 IC$_{50}$ μM	recomb. MMP-2 IC$_{50}$ μM	recomb. MMP-3 IC$_{50}$ μM	recomb. MMP-8 IC$_{50}$ μM	recomb. MMP-9 IC$_{50}$ μM	% inhibition of capillary formation at 50 μM	% inhibition of lung metastasis formation[c]
19	**s**	Et$_2$N	inactive	30	1	30	70	>100	-	-
20	**t**	Bu$_2$N	inactive	70	50	30	90	>100	-	-
21	**u**	(CH$_2$)$_5$N	inactive	70	1	90	>100	0.5	-	-
22	**v**	O(CH$_2$CH$_2$)$_2$N	inactive	50	>100	1	30	>100	-	-

[a] Standard deviations are less than 20% of the mean values. [b] Errors for these measurements are 5% of the mean values. [c] Murine melanoma, 50 mg/kg ip administration daily for 3 weeks. [d] Sodium salt. [e] Cyclohexylammonium salt. [f] Monoethyl ester lithium salt.

we examined the first carbamoylphosphonic acid, namely *N*-cyclohexylcarbamoylphosphonic acid. The potency showed (vide infra) by this compound motivated us to study systematically the MMP inhibitory potency of various classes of carbamoylphosphonic acids. The present article describes the results obtained from series of *N*-alkyl-, *N*-cycloalkyl-, and *N*,*N*-dialkylcarbamoylphosphonic acids.

Carbamoylphosphonate esters have been known for several decades.[15] In this work, we chose the synthetic approach based on the reaction of triethyl phosphonothiolformate[16] (**1**) with an amine to the respective diethyl *N*-(cyclo)alkylcarbamoylphosphonate (**2**, Scheme 1). This approach uses a common phosphorus-containing starting material, instead of necessitating the synthesis of a series of isocyanates. Triethyl phosphonothiolformate has previously been shown to react with ammonia predominantly by cleavage of *S*-acyl fission,[17] as opposed to *P*-acyl fission which is characteristic of simple acylphosphonate diesters.[11] Apparently, the EtS$^-$ anion is a better leaving group than diethyl phosphite anion. The carbamoylphosphonate diesters formed in these reactions lack the sensitivity to hydrolytic *P*-acyl cleavage shown by acylphosphonate diesters.[11] This stability is the result of the low reactivity of carbamate amide's C═O group, compared to the ketone C═O group in simple acylphosphonate diesters. The phosphonate diesters (**2**) were subsequently dealkylated by bromotrimethylsilane followed by methanol treatment to the corresponding *N*-(cyclo)alkylcarbamoylphosphonic acids (**3**). The final products were isolated either as crystalline free acids or as salts. All carbamoylphosphonic acids were characterized by elemental analysis and the customary spectroscopic methods, the results of which fully confirmed their structures and purity. The synthesis of *N*-cyclopentylcarbamoylphosphonic acid illustrates this method. Alkyl-and cycloalkylarbamoylphosphonic esters, acids and salts are stable and storable in the neat state at ambient temperature without notable decomposition during several years of this project. Cyclopentylcarbamoylphosphonic acid was kept at ambient temperature at pH 7 in D_2O for over a year with no visible change in the ^{31}P NMR spectrum. However, no special stability studies were performed.

The newly synthesized compounds belonging to series of alkyl-, cycloalkyl- and *N*,*N*-dialkylcarbamoylphosphonic acids were evaluated using three in vitro and one in vivo models, as explained in the next section. The results of these tests are presented in Table 1.

Biology

The in Vitro Invasion Assay: Chemoinvasion. Breaching of the basement membrane by tumor cells is one of the turning points that put tumor cell dissemination beyond conventional treatment. This breaching is dependent on the presence and activity of certain MMPs, mainly MMP-2 and MMP-9. Therefore, we used the traversion of a reconstituted basement membrane by tumor cells as a screen to evaluate the potential inhibitory potency of the newly synthesized molecules.[18]

All compounds have been subjected to the chemoinvasion test at three concentrations, namely 100, 50, and 10 μM. Compounds that showed no effect at 100 μM were classified as inactive and were not studied further. This assay determines the extent of invasion of tumor cells across a reconstituted membrane in the absence or in the presence of varying concentrations of potential inhibitors. The assay evaluates the effect of the drug in an environment similar to that in vivo, and therefore it has a higher predictive value than measuring inhibitory potency on recombinant enzymes. The extent of inhibition of invasion caused by the compounds at 50 μM concentration is shown in Table 1, column 4.

Determination of IC_{50} Using Recombinant Enzymes. The IC_{50} values of all compounds have been determined on five recombinant MMP subtypes, namely MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9.[19] The IC_{50} values obtained from the compounds examined are displayed in Table 1, columns 5−9.

Capillary Formation. New blood vessel formation is a critical step in the expansion and in dissemination of a given tumor. Tube formation has been shown to be dependent on cellular proliferation and on expression of certain MMPs. Therefore, this experiment is an accepted model of angiogenesis.[20] Selected compounds were tested as to their potency to inhibit capillary formation in vitro (Table 1, column 10).

The Murine Melanoma Model. Experimental metastasis formation was studied in a murine model using B16F10 melanoma tumor cells injected IV.[21] In this model, metastases are formed in the lungs of the mice. Effectiveness of potential treatment is measured by its ability to reduce the number of metastatic foci in the lungs. Most compounds that showed 50% or greater reduction in cell invasiveness at 50 μM, and submicromolar values of IC_{50} on MMP-2 were tested in vivo. The examined compounds were administered daily IP in a dose of 50 mg/kg for 3 weeks. The results obtained from the compounds tested are listed in (Table 1, column 11).

Discussion

The malignant behavior of cancer cell is mainly based on the unrestrained invasiveness of the cells, or of certain subpopulation within the given tumor and less on extensive proliferation. Radio-, photo-, and chemotherapies, the current cancer treatments, affect nonselectively mainly proliferating cells, while invasiveness remains unaffected. In malignant tumors, such antiproliferative therapy is the only possible strategy today, despite its inevitable side effects on normal proliferative cells such as the digestive tract and the immune system. The inhibition of MMPs is, therefore, an important therapeutic target, yet no MMP inhibitor has been approved for clinical use. Recently, there were several attempts to rationalize this failure.[3,22−24]

Among MMPs, gelatinases (MMP-2 and MMP-9) are especially important in connection with the processes of tumor growth, invasion, and metastasis.[23] Thus, gelatinase inhibitors have been studied extensively as new types of anticancer drugs.

There are several previous reports on phosphorus containing functions as zinc-binding groups in MMPs.[8−10] For example, some phosphonic derivatives of pseudopeptide sequences gave gelatinase-A (MMP-2) inhibitors with K_i values in the nanomolar range.[8] Other papers report on phosphinic acids of various structures.[9,10] However, none of the compounds described in these papers was reported to show any in vivo potency.

The acylphosphonic function is composed of a keto group and an adjacent phosphonic group in an arrangement ideally situated to form five-membered ring chelates. The carbonyl groups in carbamoylphosphonates may be expected to be better chelating groups than those in α-ketophosphonates, since the electron density on the carbonyl oxygens in amides is higher than in ketones, as a result of electron donation by the amide nitrogen.[25] An additional possible advantage of the carbamoyl group may connected to its improved ability to participate in hydrogen bonding with the enzyme (a) by the carbonyl of the amide because of its increased polarity and (b) by the NH group.

Although there exist reports on the interaction of acylphosphonates with calcium and on the mode of calcium binding by a bisacylphosphonate,[12] the use of carbamoylphosphonic acids in MMP inhibitors is novel. We have reported recently the results of solution studies of some carbamoylphosphonate complexes with zinc and other cations.[14] The stability order found for the complexes of cyclopentylcarbamoylphosphonic acid is Ca(II) < Mg(II) < Zn(II). In addition, the results of this study lend support to the assumption concerning the participation of the carbonyl group in metal binding, presumably through a five-membered ring chelate formation.

It is worthy of note that all carbamoylphosphonates, being dibasic acids ($pK_{a1} < 2$, $pK_{a2} \sim 5-5.5$), are fully ionized and soluble at physiological pH. The lower MW compounds are soluble in water also as the free acids. For example, 1 g of cyclopentylcarbamoylphosphonic acid, **3m** (Table 1, entry 13), dissolves in 4 mL of water. This is of considerable importance for their development as drugs, since matrix metalloproteinases reside in the intercellular space; therefore, good inhibitors should have good water solubility. For example, hydroxamate MMP inhibitors, insoluble in water, have been reported to accumulate/precipitate in various tissues, causing "musculoskeletal" side effects.[3]

Both tumor cell invasiveness and angiogenesis have been shown to be MMP-dependent. Therefore, the evaluation of the efficacy of our MMP inhibitors as potential drugs for cancer was focused on two characteristics (in addition to determining their inhibitory

Table 2. Physical Properties and NMR Spectral Data of Alkylcarbamoylphosphonate Diesters **2**[a]

	structure R in R-NHCOPO$_3$Et$_2$	mp	NMR
			Monoalkylcarbamoylphosphonate Diesters **2**
1	methyl **2a**	oil	($CDCl_3$) ^{31}P −1.18 ppm. ^{1}H, 1.27 (t, J = 7.5 Hz, 6H), 2.79 (d, J = 4.8 Hz, 3H), 1.48 (m, 4H), 7.85 (br s, 1H)
2	ethyl **2b**	oil	($CDCl_3$) ^{31}P −0.99 ppm. ^{1}H, 1.20 (td, J = 7.2, 0.9 Hz, 3H), 1.37 (td, J = 6.9, 0.6 Hz, 6H), 3.38 (quint, J = 6.9, 0.6 Hz, 2H), 4.18−4.30 (m, 4H), 7.28 (br s, 1H)
3	1-propyl **2c**	oil	($CDCl_3$) ^{31}P −0.99 ppm. ^{1}H, 0.92 (t, J = 7.2 Hz, 3H), 1.35 (t, J = 6.9 Hz,6H), 1.56 (sex., J = 7.2 Hz, 2H), 3.28 (q, J = 7.2 Hz, 2H), 4.10−4.30 (m, 4H), 7.10 (br s)
4	1-butyl **2d**	oil	($CDCl_3$) ^{31}P: −3.31 ppm (t). ^{1}H, 0.92 (t, J = 7.2 Hz, 3H), 0.36 (m, 8H), 1.52 (m, 2H), 3.32 (m, 2H), 4.15−4.23 (m, 4H), 7.22 (m, 1H)
5	Me_2CHCH_2 **2e**	oil	($CDCl_3$) ^{31}P: 1.45 ppm (quin). ^{1}H, 0.94 (d, J = 6.6 Hz, 6H), 1.37 (t, J = 7.2 Hz), 1.84 (m, 1H), 3.17−3.48 (m, 4H), 4.23 (m, 4H), 7.15 (br s, 1H)
6	$Me_2CHCH_2CH_2$ **2f**	oil	($CDCl_3$) ^{31}P: δ 1.02 ppm (t), ^{1}H, δ 0.87 (d, J = 6.7 Hz, 6H), 1.31 (t, J = 7 Hz, 6H), 1.39 (q, J = 7 Hz, 2H), 1.57 (nonet, J = 6.7 Hz, 1H), 3.29 (m, 2H), 4.17 (m, 4H), 7.32 (br s)
7	$PhCH_2CH_2$ **2g**	oil	($CDCl_3$) ^{31}P: −3.51 ppm (t), ^{1}H, 1.32 (t, J = 6.9 Hz, 6H), 2.85 (t, J = 6.9 Hz, 2H), 3.59 (q, J = 6.9 Hz, 2H), 4.15(m, 4H), 7.17−7.29(m, 5H)
8	2-propyl **2h**	oil	($CDCl_3$) ^{31}P −0.46 ppm. ^{1}H, 1.34 (td, J = 6.9, 0.6 Hz, 6H), 1.74 (d, J = 6.6 Hz, 3H), 1.77 (d, J = 6.6 Hz, 3H), 4.2 (m, 5H), 6.95 (m, 1H)
9	(*RS*)-2-butyl (2-Pr$_2$ ester) **2i**	oil	($CDCl_3$) ^{31}P −2.69 ppm. ^{1}H, 0.88 (t, J = 7.5 Hz, 3H), 1.14 (d, J = 6.6 Hz, 3H), 1.34 (d, J = 6.3 Hz, 6H), 1.32 (d, J = 6.3 Hz, 6H), 1.48 (qui, J = 6.9 Hz, 2H), 3.97 (m, 1H), 4.74 (m, 2H), 6.85 (br. s.)
10	3-pentyl **2j**	oil	($CDCl_3$) ^{31}P −0.68 ppm. ^{1}H, 0.88 (t, J = 7.2 Hz, 6H), 1.35 (t, J = 7.2 Hz, 6H), 1.41 (m, 2H), 1.57 (m, 2H), 3.85 (m, 1H), 4.21 (m, 4H) 6.80 (br.s.)
			Cycloalkylcarbamoylphosphonate Diesters
11	cyclopropyl **2k**	oil	($CDCl_3$) ^{31}P −1.59. ^{1}H, 0.60 (s, 2H), 0.81 (d, J = 6.6 Hz, 2H), 1.34 (t, J = 6.9 Hz, 6H), 2.84 (m, 1H), 4.2 (m, 4H) 7.35 (m, 1H)
12	cyclobutyl **2l**	oil	($CDCl_3$) ^{31}P −1.42. ^{1}H, 1.29 (t, J = 7.3 Hz, 6H), 1.65 (m, 2H), 1.95 (m, 2H), 2.24 (m, 2H), 4.15 (m, 4H), 4.39 (quin, J = 8.3 Hz, 1H), 7.65 (m, 1H)
13	cyclopentyl **2m**	oil	($CDCl_3$) ^{31}P −3.55. ^{1}H, 1.33 (t, J = 6.9 Hz, 6H), 1.40−1.50 (m, 2H), 1.50−1.80 (m, 4H), 1.96 (m, 2H), 4.20 (m, 5H), 7.10 (br.s. 1H)
14	cyclohexyl **2n**	oil	($CDCl_3$) ^{31}P −3.20. ^{1}H, 1.05−1.4 (m, 5H,), 1.32 (t, J = 7.2 Hz, 6H), 1.53−1.75 (m, 3H), 1.82−1.92 (m, 2H), 3.75−3.89 (m, 1H), 4.18 (m, 4H), 6.98 (b 1H)
15	cycloheptyl **2p**	mp 35−38	($CDCl_3$) ^{31}P −0.98. ^{1}H, 1.33 (t, J = 7.2 Hz, 6H), 1.40−1.97 (m, 12H), 3.98 (m, 1H), 4.18 (m, 4H), 7.03 (m, 1H)
16	cyclooctyl **2q**	oil	($CDCl_3$) ^{31}P −2.66. ^{1}H, 1.33 (d, J = 6.3 Hz, 6H), 1.31 (d, J = 6.3 Hz, 6H), 1.51 (m, 12H), 1.78 (m, 2H), 4.03 (m, 1H), 4.72 (sext, 2H)
17	cyclohexylCH_2 **2r**	oil	($CDCl_3$) ^{31}P −2.01. ^{1}H, 0.8−1.3 (m, 5H), 1.33 (t, J = 6.9 Hz, 6H), 1.4−1.75 (m, 5H), 3.13 (t, J = 6.9 Hz, 2H), 4.18 (m, 4H), 7.23 (m, 1H)
			N,*N*-Dialkylcarbamoylphosphonate Diesters
	structure of R_2N in $R_2NCOPO_3Et_2$		NMR
18	Et_2N **2s**	oil	($CDCl_3$), ^{31}P −0.35. ^{1}H, 1.16 (t, 3H), 1.26 (t, 3H), 1.39 (t, 6H), 3.41 (q, 2H), 3.75 (q, 2H), 4.18−4.25 (m, 4H)
19	Bu_2N **2t**	oil	($CDCl_3$), ^{31}P −0.33. ^{1}H, 0.86−0.93 (m, 6H), 1.26−1.36 (m, 10H), 1.46−1.62 (m, 4H), 3.30 (t, 2H), 3.60 (t, 2H), 4.17−4.23 (m, 4H)
20	$(CH_2)_5N$ **2u**	oil	($CDCl_3$), ^{31}P −0.94. ^{1}H, 1.34−1.56 (m, 12H), 3.36 (t, 2H), 3.65 (t, 2H), 4.15−4.23 (m, 4H)
21	$O(CH_2CH_2)_2N$ **2v**	oil	($CDCl_3$), ^{31}P −0.06. ^{1}H, 1.40 (m, 6H), 3.41−3.52 (m, 2H), 3.55−3.65 (m, 4H), 3.72−3.87 (m, 2H), 4.18−4.25 (m, 4H)
			Carbamoylphosphonic Acids Reported in This Paper
	structure R in R-NHCOPO$_3$H$_2$	mp	NMR
			Alkylcarbamoylphosphonic Acids
1	methyl **3a**	152−155	(DMSO-d_6) ^{31}P, −2.08. ^{1}H, 2.60 (d, J = 4.6 Hz, 3H), 8.24 (br s, 1H,). ^{13}C 25.42 (d, J = 6.87 Hz), 173.65 (d, J = 215.5 Hz)
2	ethyl **3b**	168−170	(D_2O) ^{31}P −2.78. ^{1}H, 0.94 (dt, J = 7.2, 0.9 Hz, 3H), 3.08 (dq, J = 7.2, 0.9 Hz, 2H)
3	*n*-propyl **3c**	153	(D_2O) ^{31}P −2.79 ppm. ^{1}H, 0.69 (t, J = 7.2 Hz, 3H), 1.34 (sext, J = 6.9 Hz, 2H), 3.02 (t, J = 6.9 Hz, 2H)
4	n-Bu Na^+ salt[b] **3d**	>280 dec	(D_2O) ^{31}P −2.41 (s). ^{1}H, 0.71 (td, J = 7.2, 1.9 Hz, 3H), 1.15 (sext, J = 7.2 Hz, 2H), 1.33 (q, J = 7.2 Hz, 2H), 3.06 (t, J = 7.2 Hz, 2H)
5	$Me_2CHCH_2^-$ $C_6H_{11}NH_3^+$ salt **3e**	203−6	(D_2O) ^{31}P: δ 2.02 ppm (s) ^{1}H, δ 0.9 (d, J = 6.7 Hz), 1.09−1.44 (m, 5H), 1.60−2.08 (m, 6H), 3.09 (dd, J = 6.8, 0.7 Hz, 2H)
6	$Me_2CHCH_2CH_2^-$ $C_6H_{11}NH_3^+$ salt **3f**	193−8	(D_2O) ^{31}P: δ 1.99 ppm (s) ^{1}H, δ 0.90 (d, J = 6.6 Hz, 6H), 1.10−1.50 (m, 7H), 1.62 (m, 1H), 1.65−2.05 (m's, 5H), 3.10−3.22 (m, 1H), 3.28 (t, J = 7 Hz, 2H)
7	$PhCH_2CH_2$ **3g**	>210 dec	(D_2O) ^{31}P: δ −2.7(s) ^{1}H, δ 2.71 (t, J = 7.2 Hz, 2H), 3.34 (t, J = 7.2 Hz, 2H,), 7.17−7.25 (m,5H)
8	2-propyl **3h**	150−1	(D_2O) ^{31}P −2.70 ppm. ^{1}H, 0.943 (d, J = 6.6 Hz, 3H, $CH(CH_3)_2$), 0.947 (d, J = 6.6 Hz, 3H, $CH(CH_3)_2$), 3.807 (sep, J = 6.6 Hz, 1H, C*H*)
9	(*RS*)-2-butyl **3i**	179−180	(D_2O) ^{31}P −2.71 ppm. ^{1}H, 0.65 (t, J = 7.5 Hz, 3H), 0.93 (d, J = 6.6 Hz, 3H), 1.28 (m, 2H), 3.65 (m).
10	3-pentyl **3j**	154−155	(D_2O) ^{31}P −2.64 ppm. ^{1}H, 0.68 (t, J = 7.2 Hz, 3H), 1,25 (sep, J = 7.2 Hz, 2H), 1.42 (sep, J = 7.2 Hz, 2H), 3.55 (m, 1H)

Table 2 (Continued)

	structure R in R-NHCOPO$_3$H$_2$	mp	NMR
			Cycloalkylcarbamoylphosphonic Acids
11	cyclopropyl **3k**	156−157	(D_2O) ^{31}P −3.22 ppm. ^{1}H, 0.34 (m, 2H), 0.54 (q, J = 7.2 Hz, 2H), 2.39 (m, 1H)
12	cyclobutyl **3l**	153−154	(D_2O) ^{31}P −5.27 ppm. ^{1}H, 1.43−1.52 (m, 2H), 1.75 (m, 2H), 2.0 (m, 2H), 4.01 (quin, J = 7.1 Hz, 1H)
13	cyclopentyl **3m**	163−164	(D_2O) ^{31}P, −2.73 ppm. ^{1}H, 1.22−1.47 (m 6H), 1.63−1.75 (m, 3H), 3.89 (quin, J = 6 Hz, 1H)
14	cyclohexyl **3n**	184−185	(D_2O) ^{31}P −5.27 ppm. ^{1}H, 0.85−1.15 (m, 5H), 1.3−1.6 (m, 5H), 3.45 (m, 1H)
15	cyclohexyl monoEt ester Li salt **3o**	>210 dec	($CDCl_3$) ^{31}P: −1.16. ^{1}H, 1.03−1.64 (m, 13H), 3.54 (m, 1H), 3.76 (m, 2H)
16	cycloheptyl **3p**	158−9	(D_2O) ^{31}P: −2.55 ppm. ^{1}H, 3.69 (m, 1H), 1.7−1.57 (m, 2H), 1.5−1.2 (m, 10H)
17	cyclooctyl **3q**	179−180	(D_2O) ^{31}P −2.51 ppm. ^{1}H, 1.35−1.65 (m, 14H), 3.8 (m, 1H)
18	cyclohexylmethyl **3r**	156−157	(D_2O) ^{31}P −2.65 ppm. ^{1}H, 0.6−1.5 (m, 11H), 2.88 (d, J = 6.9 Hz, 2H)
			N,*N*-Dialkylcarbamoylphosphonic Acids
19	Et_2N $COPO_3H_2$[c] **3s**	oil	(D_2O) ^{31}P −1.57. ^{1}H, 0.92 (t, 3H), 1.02 (t, 3H), 3.15−3.22 (m, 2H), 3.50−3.55 (m, 2H)
20	Bu_2N $COPO_3H_2$ **3t**	oil	(D_2O) ^{31}P −1.61. ^{1}H, 0.71−0.77 (m, 6H), 1.01−1.21 (m, 4H), 1.31−1.51 (m, 4H), 3.18 (t, 2H), 3.51 (t, 2H)
21	$(CH_2)_5NCOPO_3H_2$ **3u**	181−182	(D_2O) ^{31}P −1.46. ^{1}H, 1.36−1.58 (m, 6H), 3.37 (t, 2H), 3.67 (t, 2H)
22	$O(CH_2CH_2)_2$-$NCOPO_3H_2$ **3v**	137−138	(D_2O) ^{31}P −2.61. ^{1}H, 3.43−3.53 (m), 3.56−3.69 (m, 4H), 3.75−3.89 (m, 2H)

[a] Acceptable elemental analysis results have been obtained for all new compounds. [b] The anilinium salt of *N*-1-butylcarbamoylphosphonic acid has been reported: Sekine, M.; Yamagata, H.; Hata, T. A general and conventient method for the synthesis of unesterified carbamoyl and thiocarbamoylphosphonic acids. *Tetrahedron Lett.* **1979**, 3013−3016. [c] The anilinium salt of *N*,*N*-diethylcarbamoylphosphonic acid has been reported: Morita, T.; Okamoto, Y.; Sakurai, H. Dealkylation reaction of acetals, phosphonate and phosphate esters with chlorotrimethylsilane/metal halid reagent in acetonitrile and its application to the synthesis of phosphonic acids and vinyl phosphates. *Bull Chem. Soc. Jpn.* **1981**, *54*, 267−273.

potency on recombinant enzymes), namely, appraising their efficacy as drugs preventing (a) cellular invasiveness and (b) angiogenesis, two processes crucial for tumor growth and dissemination and both dependent on gelatinases (MMP-2 and MMP-9).[20]

Finally, compounds showed promising activity in the in vitro murine melanoma model. Examination of the results displayed in Table 1 led to the following conclusions:

1. Most compounds show considerable selectivity for MMP-2 over the other MMP subtypes examined.

2. Cycloalkylcarbamoylphosphonic acids are significantly more potent, in most cases, toward MMP-2 than comparable open-chain alkyl derivatives. This is apparent from the comparison of the cyclopropyl derivative, **3k** (Table 1, entry 11) with the 1-propyl and 2-propyl compounds (**3c** and **3h**, Table 1, entries 3 and 8, respectively), as well as the cyclobutyl (**3l**, Table 1, entry 12) with 1-butyl and 2-butyl derivatives (**3d** and **3i**, Table 1, entries 4 and 9, respectively), and finally from the comparison of the cyclopentyl (**3m**, Table 1, entry 13) with 3-pentylcarbamoylphosphonic acid (**3j**, Table 1, entry 10).

3. The systematic variation of the ring size in cycloalkylcarbamoylphosphonates from three to eight led to conclusions regarding the effect of the ring size on the inhibitory potency, specificity, and selectivity. Optimal activity against MMP-2 was shown by cyclopentylcarbamoylphosphonic acid, **3m** (Table 1, entry 13). Interestingly, the lower homologous cyclopropyl and cyclobutyl derivatives **3k** and **3l** (Table 1, entries 11 and 12, respectively) were more potent against MMP-8, while the higher homologous cyclohexyl derivative **3n** (Table 1, entry 14) was most active against MMP-1 with significant selectivity. The latter compounds may serve as leads for the design of selective inhibitors for the other enzymes).

4. Comparison of **3n** with **3o** (Table 1, entries 14 and 15) shows that the optimal biological activity against MMP-2 requires the presence of two acidic protons in the phosphonic function.

5. There is no simple correlation between the activity shown in the invasion assay and that in the inhibition assay of the MMP-2, with in vivo activity. Our results indicate that neither of them alone is a reliable predictor of cancer related to in vivo activity. Conversely, lack of potency in the invasion assay can serve as an indicator of potency on enzymes MMP-2, and perhaps MMP-9, but not on other MMP subtypes, as apparent from Table 1, entries 5, 11, 12, and 14).

6. *N*,*N*-Dialkylcarbamoylphosphonates **3s**, **3t**, **3u**, and **3v** (Table 1, entries 19−22) showed low or no activity. This may be related to the lack of NH in the molecules and may support the assumption regarding the importance of hydrogen bond interaction between the inhibitor and the enzyme.

Conclusion

A large body of evidence has linked the pathology of certain diseases with the overexpression of MMPs. In this study, we show that several alkyl- and cycloalkylcarbamoylphosphonic acids inhibit selectively in vitro MMP-2 and are effective in preventing tumor cell dissemination in vivo. Further, some of our compounds have been shown to possess significant antiangiogenic activity, which enhances the anticancer effect of the drugs. *N*-Cyclopentylcarbamoylphosphonic acid, **3m** (Table 1, entry 13), was found to be the most active compound in the series studied in this work. This compound had an IC_{50} of 80nM on MMP-2 in addition to selectivity and impressive in vivo activity. The compounds examined showed toxic effects neither in vitro nor in vivo in the concentrations used.

Experimental Section

***N*-Cyclopentylcarbamoylphosphonic Acid. (a) Diethyl *N*-Cyclopentylcarbamoylphosphonate.** To a solution of triethyl phosphonothiolformate (1.97 g) in acetonitrile (15 mL)

was added cyclopentylamine (0.82 g), and the solution was kept at room-temperature overnight. The volatiles were removed, and the residue was dried in high vacuum to yield a yellow oil, which was purified by chromatography over silica gel (EtOAc) to yield 1.43 g (69.5%) of product.

The physical and spectral data of this and other compounds reported in this paper are summarized in Table 2.

(b) *N*-Cyclopentylcarbamoylphosphonic Acid. A solution of diethyl cyclopentylcarbamoylphosphonate obtained in the previous step (1.02 g, 3.46 mmol) and bromotrimethylsilane (2.24 mL, 17.3 mmol) in MeCN (10 mL) was kept at room-temperature overnight. After the addition of MeOH (5 mL), the volatiles were removed in vacuo to leave a crystalline residue (0.92 g), which was recrystallized from EtOH; mp 163−164 °C.

1. In Vitro Invasion Assay: Chemoinvasion. Boyden chamber chemoinvasion assays were performed as previously described.[18] Matrigel (25 μg) was dried on a polycarbonate filter (PVP free, Nucleopore). Fibroblast-conditioned medium (obtained from confluent NIH-3T3 cells cultured in serum free DMEM) was used as the chemoattractant. Cells were harvested by brief exposure to 1 mM EDTA, washed with DMEM containing 0.1% bovine serum albumin, and added to the Boyden chamber (200 000 cells). The chambers were incubated in a humidified incubator at 37 °C in 5% CO_2/95% air atmosphere for 6 h. The cells that traversed the Matrigel layer and attached to the lower surface of the filter were stained with Diff Quick (American Scientific Products) and counted. The results in column 4 of the Table 1 show the % reduction of the number of cancer cells that crossed the membrane, relative to the control by the examined compound at the concentration of 50 μM.

2. Chemotaxis. Since the chemoinvasion assay depends on proper migration of the cells, it was necessary to evaluate the chemotaxis of the cancer cells. This was performed in a similar way to basement membrane invasion, with the exception that the filters were coated with 5 μg of collagen IV instead of Matrigel. This amount of collagen does not form a barrier to the migrating cells but rather an attachment substratum. The results of these experiments excluded a possible inhibitory effect of the drugs on cellular motility.

3. Analysis of MMP Activity Use of Recombinant MMPs and Relevant Substrates. Commercial recombinant MMPs (R&D Systems, Minneapolis, MN) were incubated at four different concentrations (1−50 ng) with their respective substrates for 3 h. The examined compounds were added at four to six different concentrations (0.1−100 μM) to the recombinant enzymes, and the inhibitory potencies expressed in a colorimetric change were measured by an ELISA reader. The inhibitory potency (IC_{50}) was calculated from the kinetic data obtained.

4. Endothelial Capillary Tube Formation. Endothelial cells were plated on top of a thin layer of Matrigel in the presence of an angiogenic factor (bFGF/VEGF) and incubated for 4−6 h. In this time period, the endothelial cells formed tubelike structures, which resemble the tube organization of endothelial cells in vivo. The various compounds were added to the culture at five concentrations (100, 50, 25, 10, and 5 μM), and the extent of tube formation was measured by an image analysis system (Table 1, column 10).

5. Tumor Growth and Metastasis in an Animal Model. Experimental metastasis was studied in the murine melanoma model. In this model, 50 000 B16F10 tumor cells were injected into the tail vein. The mice were treated for three weeks by daily injections of 50 mg/kg of the tested drug. The mice were sacrificed after 21 days, and the tumors formed on the lungs of were counted after appropriate fixation (Table 1, column 11).

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