Carbamoylphosphonate Matrix Metalloproteinase Inhibitors 6: *cis*-2-Aminocyclohexylcarbamoylphosphonic Acid, A Novel Orally Active Antimetastatic Matrix Metalloproteinase-2 Selective Inhibitor—Synthesis and Pharmacodynamic and Pharmacokinetic Analysis

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cis-2-Aminocyclohexylcarbamoylphosphonic acid (*cis*-ACCP) was evaluated in vitro and in two in vivo cancer metastasis models. It reduced metastasis formation in mice by ~90% when administered by a repetitive once daily dosing regimen of 50 mg/kg via oral or intraperitoneal routes and was nontoxic up to 500 mg/kg, following intraperitoneal administration daily for two weeks. Pharmacokinetic investigation of *cis*-ACCP in rats revealed distribution restricted into the extracellular fluid, which is the site of action for the antimetastatic activity and rapid elimination ($t_{1/2}$ ~ 19 min) from blood. Sustained and prolonged absorption ($t_{1/2}$ ~126 min) occurred via paracellular mechanism along the small and large intestine with overall bioavailability of 0.3%. The in vivo concentrations of *cis*-ACCP in the blood in rats was above the minimal concentration for antimetastatic/MMP-inhibitory activity, thus explaining the prolonged action following once daily administration. Finally, 84% of the intravenously administered *cis*-ACCP to rats was excreted intact in the urine.

Introduction

Matrix metalloproteinases (MMPs) play a crucial role in physiological tissue remodeling and repair. However, their overexpression has been associated with a variety of chronic diseases including cancer, arthritis, osteoporosis, multiple sclerosis, arteriosclerosis, restenosis, meningitis, congestive heart failure, chronic obstructive pulmonary disease, chronic wounds, liver cirrhosis, cerebral ischemia, and others.[1] The efficient inhibition of MMPs is therefore an important therapeutic target that has attracted considerable attention within the research community for the last two decades. Yet, in spite of the huge effort devoted to this goal, no clinically useful inhibitor has been achieved, and the only MMP inhibitors in use are some chemically modified tetracyclines applied in periodontal disease.[2]

Previously, we have reported the MMP inhibitory activity of series of alkyl- and cycloalkylcarbamoylphosphonates[3] and, in more depth, the in vivo antimetastatic and antiangiogenic activity of cyclopentylcarbamoylphosphonic acid.[4] Parallel, we have found that introduction of amino groups into alkylcarbamoylphosphonates enhances their zinc binding and MMP inhibiting potency.[5] Therefore, to combine the two structural features, (a) an alicyclic ring and (b) an amino group, in one molecule, we have decided to synthesize aminocycloalkylcarbamoylphosphonates and to examine their biological characteristics. This paper describes the synthesis and results of in vitro and in vivo biological properties of such a molecule, namely, *cis*-2-aminocyclohexylcarbamoylphosphonic acid (*cis*-ACCP). The *cis*-isomer was chosen after the preliminary examination of the two geometric isomers (*cis*-ACCP and *trans*-ACCP) has revealed that only the *cis*-isomer has significant biological activity relevant to the metastatic process. Therefore, the aim of this paper is to describe the synthesis, the in vitro and in vivo antimetastatic activity, and the pharmacokinetic profile of *cis*-ACCP. There is a particular interest to characterize the effect of different modes of administration of *cis*-ACCP concentration—time profiles and to integrate them with the pharmacodynamics (i.e., the concentration—effect relationship) to be able to predict the kinetics of action following administration by the different routes, including oral, intraperitoneal, or intravenous.

Results

Chemistry. Synthesis of *cis*- and *trans*-ACCP. The syntheses of the target compounds have been accomplished using the approach reported in the previous papers[3,5] and outlined in Scheme 1. By this approach, the carbamoylphosphonic group is constructed by attaching the phosphonoformyl group to an amino group, using triethyl phosphonothiolformate. In the present case, the reactions were carried out by slow addition of phosphonothiolformate to the starting materials, which contained two primary amino groups. This way, the product mixtures obtained contained mainly (83%) the desired monophosphonoformamide in the case of the *cis*-isomer but only 28% of it in the case of the *trans*-isomer, along with the unwanted bisphosphonoformylation products. The predominant (72%) formation of the 1,2-bisphosphonoformamide in the case of the *trans*-isomer versus only 28% in the *cis*-isomer is promoted by the reduced steric hindrance in the *trans*-isomer. The reaction mixtures were separated by chromatography to isolate the pure carbamoylphosphonic acids. The products' structures were confirmed by standard analytical and spectroscopic methods.

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Scheme 1

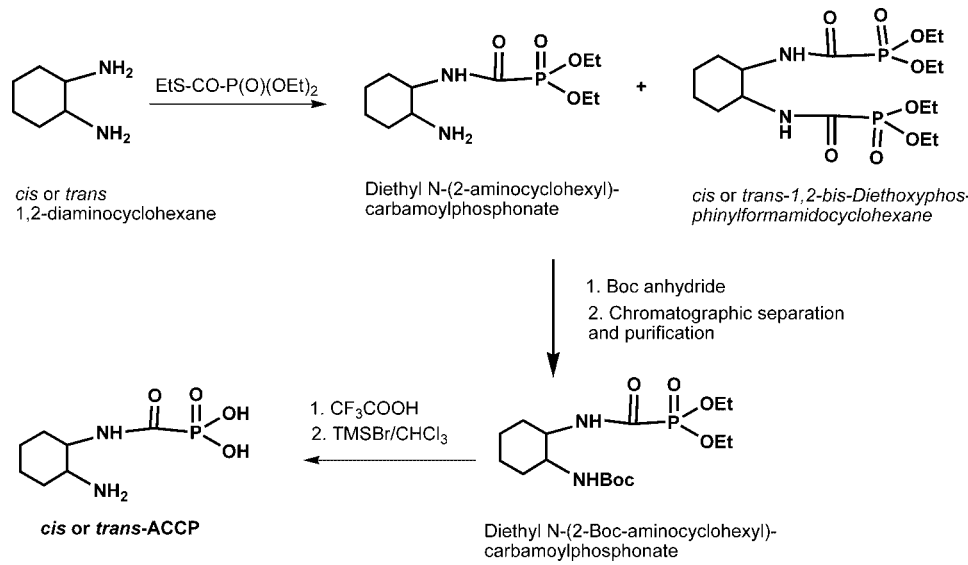

Table 1. Effect of *cis*-ACCP on Human Recombinant MMP Enzymes[a]

cmpd	IC_{50} (μM) MMP-1	IC_{50} (μM) MMP-2	IC_{50} (μM) MMP-3	IC_{50} (μM) MMP-8	IC_{50} (μM) MMP-9	IC_{50} (μM) MMP-12	IC_{50} (μM) MMP-13	IC_{50} (μM) TACE
cis-ACCP	>100	4	>100	>100	20	>100	>100	>100
trans-ACCP	90	>100	25	50	>100	>100	55	>100

[a] The enzymatic activity of the various human recombinant enzymes was measured in the presence of *cis*- or *trans*-ACCP, and the IC_{50} values were calculated. The values were obtained from two different independent measurements where the errors were 5% of the mean values.

Scheme 1 illustrates the steps of the synthetic approach used for both ACCP isomers.[6]

In silico docking studies of the enantiomers of *cis*- and *trans*-ACCP are in progress. The results will be reported separately.[7]

Biological Activity. Kinetic Parameters. The initial enzyme kinetic values of our carbamoylphosphonate inhibitors in this and previous works[3–5] were determined using a recombinant MMP-2 obtained from Dr. W. G. Stetler-Stevenson of NIH. Because this preparation is not available anymore, we had to switch to a commercially available enzyme that turned out to be less active. By using this commercial recombinant MMP-2 enzyme, different enzyme kinetic values and IC_{50} values higher by several folds were obtained. Similar kinds of changes in kinetic values were observed also by others using a specific inhibitor on MMP enzymes from different sources.[8,9] We extended our previous examinations and measured the duration of the inhibition by our compounds and have found that most previously reported carbamoylphosphonates are bound weakly to MMPs and do not inhibit the enzyme beyond about 30 min (see Figure S1, Supporting Information). Using the Lineweaver–Burk equation, we found that the mode of inhibition of MMP-2 by *cis*-ACCP is reversible and competitive in nature (see Figure S2, Supporting Information).

The specificity of the ACCP compounds was evaluated against several human recombinant enzymes. We found that *cis*-ACCP preferentially inhibits the gelatinases, that is, MMP-2 and MMP-9, with a preference for MMP-2 (IC_{50} = 4 uM). The *trans*-ACCP did not inhibit the gelatinases but had moderate activity against MMP-3 and MMP-13. These results indicate specificity of the compounds regarding binding to the enzymes (see Table 1).

Cell-Based Activity. Tumor cell invasion through a reconstituted basement membrane (Matrigel) depends on the ability of the cells to locally degrade collagen IV present in Matrigel. This degradation is MMP-2 dependent.[10] Addition of *cis*-ACCP to tumor cells prevented their traversion in a dose-dependent manner, about 90% at the highest concentration (100 uM) tested. The inhibition of the invasion of tumor cells across Matrigel-coated filters was observed concomitantly with inhibition of MMP-2 activity in the same assay and shown in Figure 1.

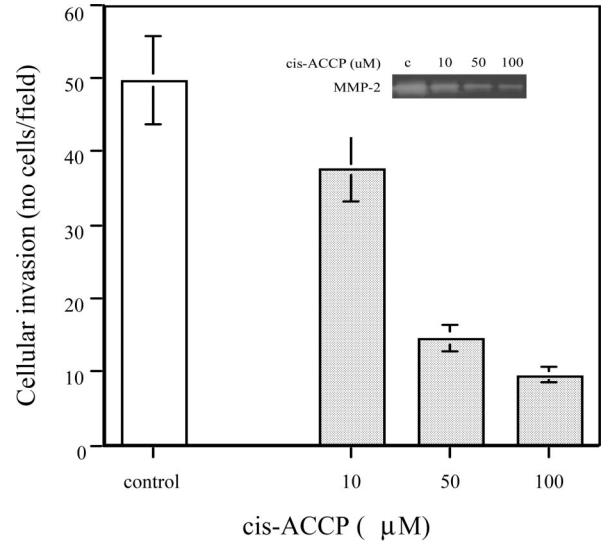

Figure 1. Effect of *cis*-ACCP on tumor cell invasion of matrigel-coated membranes in vitro. A dose-dependent inhibition is observed concomitantly with a dose-dependent inhibition of MMP-2 activity as detected in the invasion chamber (see insert). The results presented are the mean of three independent repetitions.

In Vivo Studies. Acute Toxicity. The acute toxicity of our compound was evaluated in C57Bl mice in doses of 50, 250, and 500 mg/kg intraperitoneally daily for two weeks. No toxic effects were observed in any of the treated groups. Neither have any changes been observed in the behavioral or nutritional status of the treated mice.

Table 2. Metastasis Formation in *cis*-ACCP-Treated Mice[a]

treatment	untreated	IP	oral
tumor take	8/8	6/8	2/8
No. of foci	26–35	0–20	0–10
median	31	4	1
mean	30	9	2

[a] The mice were treated with 50 mg/kg *cis*-ACCP daily for three weeks, except weekends. *cis*-ACCP was given ip or orally. After 21 days, the lungs were examined and the metastatic loci counted. In each experimental group, there were eight mice.

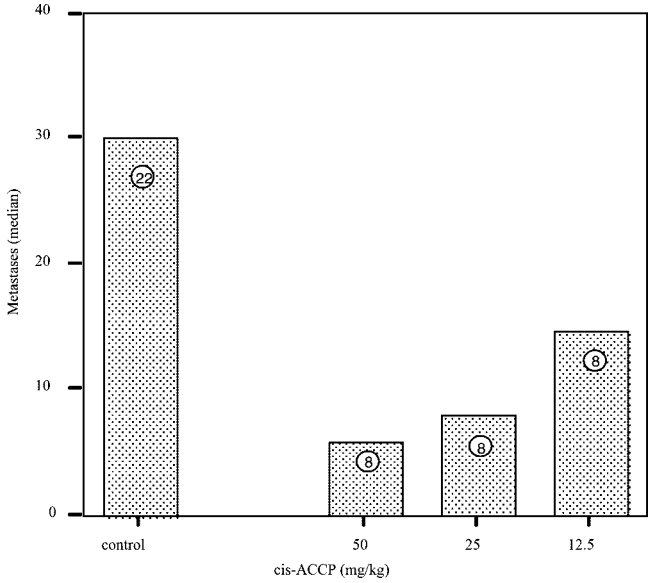

Figure 2. Effect of daily dose of *cis*-ACCP on metastases formation in mice. Mice received daily the indicated doses ip, and the lungs were examined 21 days later for metastatic loci. In each experimental group, there were eight mice.

Cancer Models.[11] The ability of *cis*-ACCP to inhibit tumor dissemination has been tested in two experimental models. Similarly to previous studies that have used hydroxamate-based inhibitors in doses of 50 mg/kg in their in vivo evaluations, we have also used the same treating dose.

Murine Melanoma Model. B16F10 murine melanoma cells (50k) were injected into the tail vein of female C57Bl mice. Simultaneously, treatment of the mice had been initiated using daily doses (except for weekends) of 50 mg/kg *cis*-ACCP to two separate groups of mice. One group was given intraperitoneal injections (ip), while the second group received oral doses, for three weeks. Both the orally and the intraperitoneally treated mice showed a significant reduction in tumor colonization following *cis*-ACCP treatment (see Table 2).

A dose–response relationship study of the antimetastatic activity of *cis*-ACCP on mice was performed by intraperitoneally administering doses ranging from 12.5 to 50 mg/kg of the drug. Upon sacrifice, the lungs were examined for metastatic loci. The results presented in Figure 2 show 50–85% inhibition of metastasis formation, depending on the dose.

Orthotopic Human Prostate Tumor Model. In the second in vivo experiment we used an orthotopic human prostate tumor model in severe combined immunodeficiency (SCID). In this model, the tumor cells were initially transfected with the luciferase gene as a reporter marker and injected into the mouse prostate. A CCCD camera, using luciferin as a detecting device, monitored tumor growth and tumor dissemination weekly. The mice were treated for six weeks by daily ip injections of 50 mg/kg of the tested drug, except for weekends. Our results indicate that *cis*-ACCP reduced both local tumor growth (see Figure 3, Parts A and B) by more then 60% and metastasis formation (see Figure 3, Part C) by more then 90%.

Pharmacokinetics and Disposition of *cis*-ACCP. The mean plasma concentrations versus time profile obtained following intravenous administration of *cis*-ACCP (50 mg/kg) to rats and the calculated pharmacokinetic parameters are presented in Figure 4 and Table 3, respectively. The major findings are the short elimination half-life and the volume of distribution that indicates permeation of *cis*-ACCP into the extracellular fluids (but not into cells).

The mean plasma concentrations versus time of *cis*-ACCP obtained following oral administration of *cis*-ACCP (150 mg/kg) to rats and the calculated pharmacokinetic parameters are shown in Figure 5 and Table 4, respectively.

Because the oral absorption seems to be a prolonged process, the permeability of *cis*-ACCP through different segments of rat intestinal wall has been evaluated in Ussing diffusion chambers. It was found that, although the rate of *cis*-ACCP passage through the different segments of rat intestine is rather slow ($P_{app} = \sim 10^{-6}$ cm/sec), the compound is evenly absorbed from all parts of the gastrointestinal tract, including the colon, as indicated by the obtained P_{app} values (Table 4).

The rate of *cis*-ACCP permeability was found to be comparable to atenolol and mannitol (in the jejunum), as seen in Figure 7. This indicates that *cis*-ACCP is absorbed mainly by a paracellular mechanism. The same permeability properties have also been found in mice intestine (data not shown).

The mean plasma concentrations versus time of *cis*-ACCP obtained following intraperitoneal administration of 150 mg/mL to rats and the calculated pharmacokinetic parameters are presented in Figure 6 and Table 5, respectively.

As the pharmacological activity of *cis*-ACCP was tested in mice, while the basic pharmacokinetic tests were done in rats, in which larger volumes of blood samples can be obtained (enabling more accurate pharmacokinetic analysis), we performed a "bridging" pharmacokinetic study in mice to ascertain the similarity between mice and rats. The mean plasma concentrations and pharmacokinetic profile of *cis*-ACCP obtained following oral administration (150 mg/kg) to mice are illustrated in Figure 8, whereas the pharmacokinetic profile of *cis*-ACCP obtained following intraperitoneal administration (50 mg/kg) to mice is illustrated in Figure 9.

The pharmacodynamics of *cis*-ACCP (i.e., concentration−effect relationship) has been found to be nonlinear. The magnitude of inhibition (i.e., reduction of enzyme activity and tumor cell invasion) is concentration-dependent up to a certain concentration. This concentration is defined in this work as MIC, which was calculated to be 12 ng/mL (50 μM) in the in vitro inhibition studies. This concentration is at least 10-fold above IC_{50} value and it causes above 70% inhibition of the cellular invasion in vitro. It is assumed that any *cis*-ACCP concentration that exceeds this value will provide the maximal antimetastatic/MMP-inhibitory activity. Thus, the maximal inhibition of MMP-2 will be retained as long as the concentrations in the blood and the extracellular fluid are above MIC. This explains the prolonged action observed following single daily administrations either orally or intraperitoneally. It also allows predicting the kinetics of action of this MMP inhibitor following various modes of administration based on the concentration−time profile that would be obtained.

The pharmacokinetic profiles described above show that in all the various routes of administration tested, the *cis*-ACCP blood concentrations attained are well above the MIC. This is

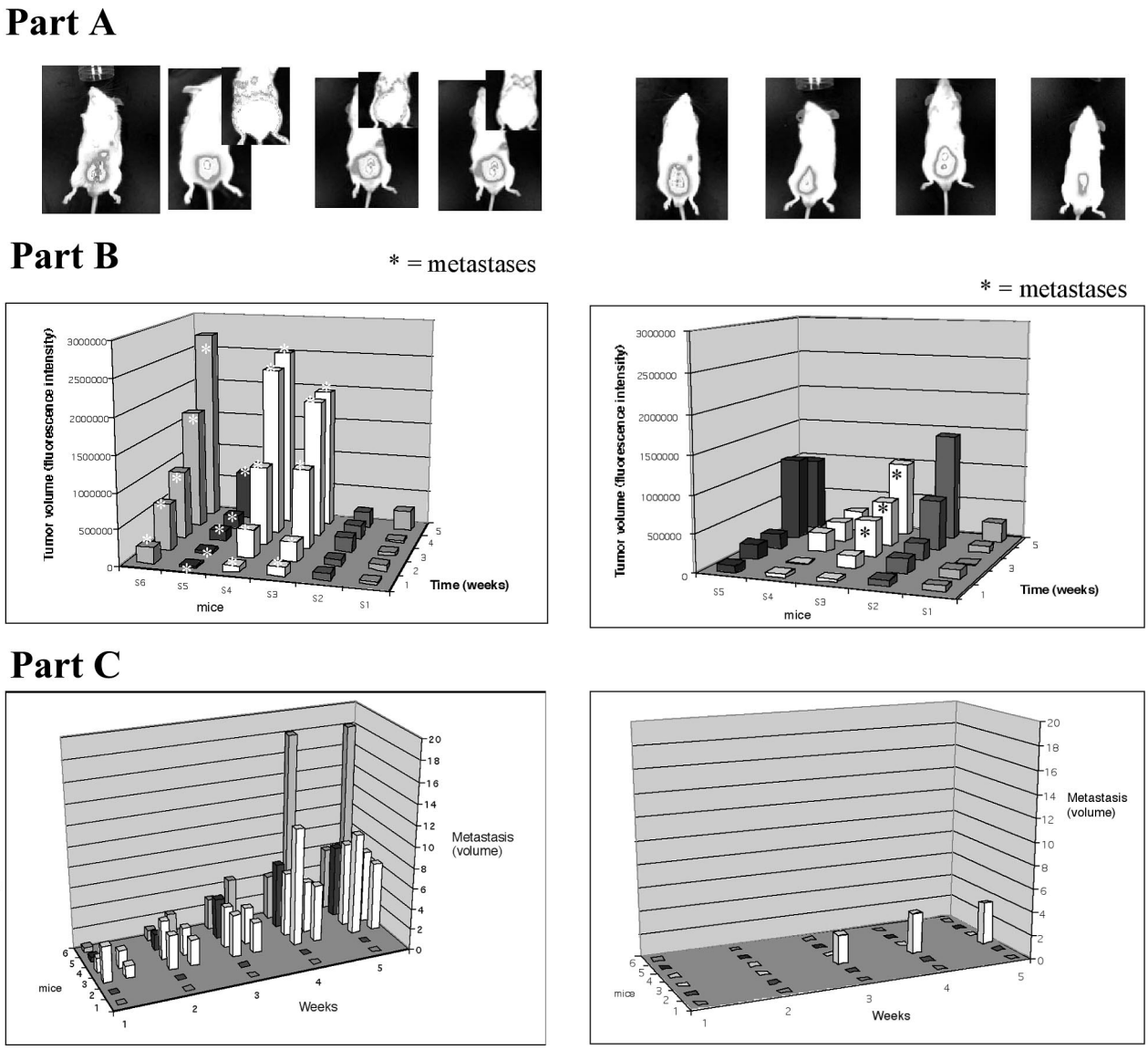

Figure 3. Effect of *cis*-ACCP on tumor size and metastasis formation in an orthotopic human prostate model in SCID mice. Human prostate tumor cells were injected directly into the prostate of SCID mice and tumor growth and metastasis formation were monitored weekly by a CCD camera. Part A shows the tumor and metastasis reflection in representative mice of control and treated mice; Part B shows the weekly tumor growth as calculated from the amount of luminescence of control and treated mice; Part C shows the relative size of the metastatic loci in control and treated mice.

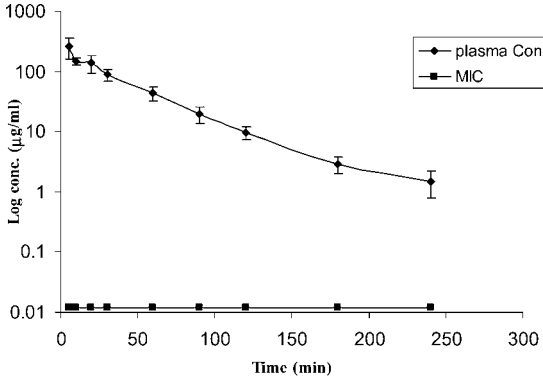

Figure 4. Logarithmic illustration of mean plasma concentration (µg/ml) of *cis*-ACCP obtained following intravenous administration (50 mg/kg) of *cis*-ACCP to five rats.

demonstrated by the MIC values that have been added to all the PK figures in this article.

Table 3. Mean Pharmacokinetic Parameters of *cis*-ACCP Obtained Following Intravenous (50 mg/kg) Oral (150 mg/kg) and Intraperitoneal (150 mg/kg) Administration of *cis*-ACCP to Five Rats[a]

PK parameter	iv	PO	ip
AUC (min·µg/mL)	7637	79	2677
$t_{1/2}$ (min)	18.8	126	108
C_{max} (ng/mL)	306000	712	144000
CL (mL/min/kg)	6.9	–	–
T_{max} (min)	–	17.4	0.5
V_{ss} (mL/Kg)	186	–	–
F (%)	–	0.35	11.8
Fu (%)	83.9	2.2	48
Ttime over MIC (hr)	>4	>6	>24

[a] AUC, area under the plasma concentration vs time curve; $t_{1/2}$, half-life; C_{max}, maximal plasma concentration; CL, clearance; T_{max}, time to maximal concentration; V_{ss}, steady state volume of distribution; *F*, bioavailability; Fu, fraction excreted unchanged in the urine; MIC, minimal inhibitory concentration.

To obtain a clearer picture of the bioavailability of *cis*-ACCP, we determined the total amount excreted unchanged in rat urine during 48 h following administration by the different routes.

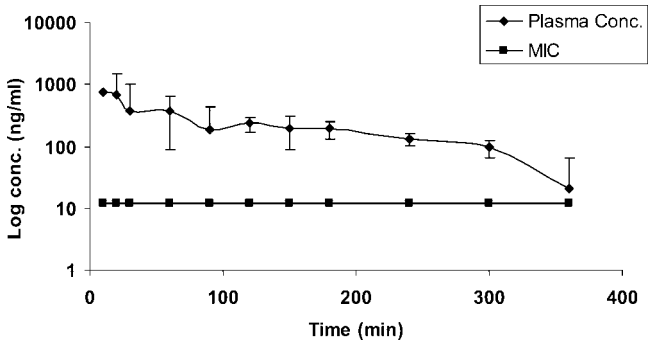

Figure 5. Logarithmic illustration of mean plasma concentration (ng/mL) of *cis*-ACCP obtained following oral administration (150 mg/kg) of *cis*-ACCP to five rats versus MIC.

Table 4. P_{app} Values Obtained for *cis*-ACCP in the Differed Segments of Rat and Mice Intestine in the Ussing Chamber

	rats		mice	
intestine segment	P_{app}	SD	P_{app}	SD
jejunum	7.90E−6	3.63E−6	8.3E−6	2E−6
ileum	7.01E−6	3.03E−6	–	–
colon	9.27E−6	1.78E−6	2.4E−6	8.4E−7

Table 5. Pharmacokinetic Parameters of *cis*-ACCP Obtained following Oral Administration of *cis*-ACCP (150 mg/kg) to Mice

PK parameter	t_{max} (hr)	C_{max} (μg/mL)	AUC[a] (hr·μg/mL)	$t_{1/2}$ (min)	time over MIC[a] (hr)
value	1	0.544	4.5	811[a]	>12

[a] Sampling lasted for 12 h.

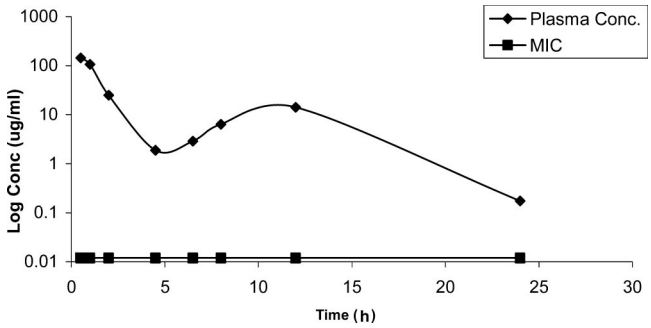

Figure 6. Logarithmic illustration of mean plasma concentration (μg/mL) of *cis*-ACCP obtained following ip administration (150 mg/kg) to four rats.

The data presented in Table 3 show that 84% of the intravenously administered drug are excreted unchanged.

Discussion

Because the wide ranging and severe implications resulting from the overexpression of MMPs have been recognized, the design of clinically useful inhibitors has become an important goal in many academic and industrial laboratories. Following the lesson learned from the inhibitor design for angiotensin converting enzyme (another zinc peptidase), the design of MMP inhibitors was mostly based on a zinc binding group connected to peptidomimetic or similar side chains. These efforts have been focused on finding the most in vitro potent inhibitors possible. From the early days of the collagenase research, the hydroxamic group has proved to be the most potent ZBG and, therefore, most inhibitors have been based on it.

Indeed, the hydroxamate-based MMP inhibitors have been found the most in vitro powerful ones that exist and, thus, should

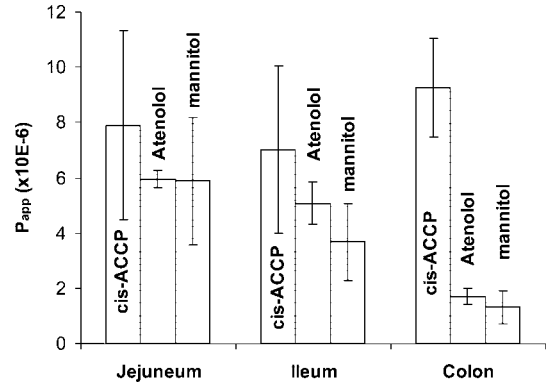

Figure 7. P_{app} values for *cis*-ACCP in the different intestine segments of rats compared to the P_{app} values of atenolol and mannitol.

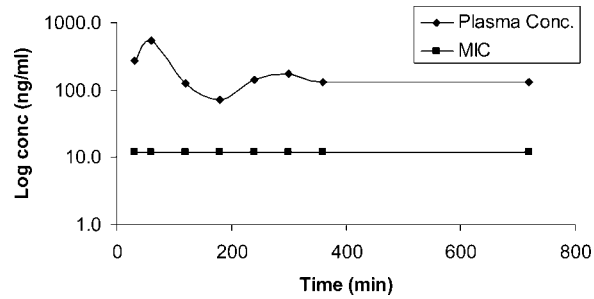

Figure 8. Logarithmic illustration of plasma concentrations (ng/mL) of *cis*-ACCP obtained following oral administration (150 mg/kg) of *cis*-ACCP to mice.

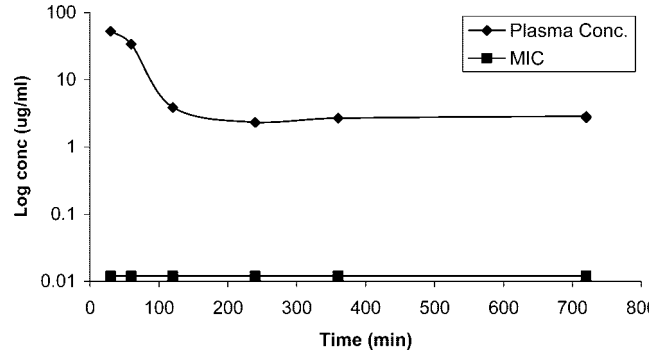

Figure 9. Logarithmic illustration of plasma concentrations (μg/mL) of *cis*-ACCP obtained following intraperitoneal administration (50 mg/kg) to mice.

have yielded the best drugs. However, so far, all attempts to find a clinically successful MMP inhibitor have failed. One of the possible reasons for the failure is probably linked to low MMP enzyme subtype selectivity. In connection with this it has been pointed out by others[12] and by us[13] that the use of such highly potent zinc-binding groups might lead to a limitation in finding specific inhibitors for the different metalloproteinases. Thus, we have proposed[13] that if one wishes to design selective MMP inhibitors, the direction to go is to seek weaker rather than stronger zinc binding groups that would leave more of the binding to the rest of the molecule by exploiting the differences between the various enzyme subtypes rather than focus mainly on the common feature, namely, the Zn^{2+} ion.

Another possible reason for the failure of the potent hydroxamate MMP inhibitors may be linked to the nonselective transition metal binding by this functional group. Hydroxamic acids have been shown to form complexes with Fe^{3+} with stability constants higher by 10^6-10^{10} than with Zn^{2+}.[14] Thus,

prolonged use of hydroxamates might remove vital iron ions from the organism that in turn might have severe consequences.

Carbamoylphosphonates (CPOs) have been reported as capable of forming zinc complexes in aqueous solution and, therefore, have been tested as potential inhibitors of zinc containing MMPs.[5] The stability constants of the zinc complexes of CPOs are much lower that those of hydroxamic acids, therefore, CPOs are not expected to lead to very potent inhibitors, yet their in vivo and pseudo in vivo potencies are considerable, in spite of their relatively high IC_{50} values. Enzyme kinetic analysis revealed that *cis*-ACCP is a reversible and competitive inhibitor.

The dianionic aminocarbamoylphosphonic acids, such as *cis*-ACCP, have a single net negative charge, and they cannot penetrate into cells. This characteristic should contribute to their nontoxic nature. These features of the compound together with its moderate inhibitory activity are essential for long-term chronic treatment, as is the case with treating cancer cell dissemination.

The daily administration of *cis*-ACCP in doses of 50, 250, and 500 mg/kg for two weeks elicited no manifestation of toxic effects in the experimental mice. Similarly, no toxic effects and no changes in the behavioral or nutritional status of the treated mice have appeared in the orthotopic human prostate tumor model experiment, in which the animals were treated for six weeks. These results allow drawing the conclusion regarding the lack of acute toxicity and may be viewed as a preliminary limited chronic toxicity results. This is of utmost importance for a drug to be used chronically, and may be connected to its short duration of binding to the enzyme and to its good water solubility. It should be mentioned that most MMP inhibitors that reached late phases of clinical testing did not fail due to their lack of activity but due to the unacceptable side effects they caused.

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 the first meaningful measurement of the biological activity of *cis*-ACCP. The results of this test have shown that the compound inhibited the invasion of tumor cells across Matrigel coated filters, in a dose-dependent manner, about 90% inhibition at the highest dose used.

The cell-associated inhibitory concentrations were higher than those presented for isolated recombinant enzymes, most likely because of the cellular environment of the invasion assay, namely, ongoing synthesis of the relevant MMP and nonspecific binding or neutralization of the inhibitor. Exposure of the tumor cells to concentrations up to 200 μM of *cis*-ACCP had no effect on cellular proliferation, as monitored for 72 h (not shown).

Animal models are critical for the development of novel therapeutics.[11] The ability of *cis*-ACCP in reducing tumor dissemination was tested in a murine syngeneic melanoma model in which tumor cells (50k) had been injected into the tail vein of the mice, followed by oral or intraperitoneal administration of the drug. This model is used frequently as a rapid evaluation of drug activity. The results obtained show a very dramatic reduction in metastasis formation in both the intraperitoneally and the orally treated groups of mice, 85 and 95%, respectively (see Table 2). It was also found that there was a dose-dependent reduction of metastatic loci when the compound was administered in lower intraperitoneal doses (12.5–50 mg/kg), resulting in 50 to 85% inhibition, respectively (see Figure 2).

Compounds that have been found active in the primary in vivo screening are usually subjected to a secondary, more rigorous test such as an orthotopic implantation of human tumor cells into severe combined immunodeficiency (SCID) mice in which local growth and distant metastasis formation can be evaluated. Successful performance in this model may predict a better outcome in human patients. When the compound was tested in this model of orthotopic implantation for six weeks by daily administration (intraperitoneal injections) of 50 mg/kg, the results obtained showed that *cis*-ACCP reduced local tumor growth by about 60% and metastasis formation by about 90% (see Figure 3).

The pharmacokinetic investigation of *cis*-ACCP showed a volume of distribution of 186 mL/kg, corresponding to the volume of the extracellular fluid in rat, and indicating that that is where the drug is mainly distributed. This is an extremely important characteristic of *cis*-ACCP because it assures that the drug can easily reach its target, the MMP enzyme, which also resides in the extracellular fluids. In this respect carbamoylphosphonates are unique among MMP inhibitors, most of which are hydrophobic and water-insoluble molecules,[1] and have failed in clinical trials. Because *cis*-ACCP is a polar hydrophilic compound, its binding to serum plasma proteins is expected to be very low. Thus, the plasma concentration may also represent the *cis*-ACCP in the extracellular fluid, which happens to be in rapid equilibrium with the free (unbound) concentrations in the blood.

The results obtained in this investigation indicate that the drug concentration in the extracellular fluid significantly exceeds the minimal inhibitory concentration (MIC) of *cis*-ACCP. This is important in terms of direct correlation that may be drawn between the MIC value determined under in vitro conditions and the drug concentration in the plasma. A reasonable analogy can be made between the pharmacodynamics of *cis*-ACCP and that of beta-lactam antibiotics, which are known to have nonconcentration-dependent pharmacodynamics.[15] The MIC in the case of these antibiotics is also an in vitro parameter, and the clinical success of the treatment depends upon the duration throughout which the antibiotic concentration in the blood is maintained above MIC.[16]

Another important pharmacokinetic parameter that was revealed by the intravenous administration is the fast elimination of *cis*-ACCP from the plasma, with a mean half-life of 18.8 min. Such a rapid rate of elimination raises the question as to how a single daily dose could provide prolonged concentration above the MIC to yield efficacy, as usually 4–5 half-lives (about 1.5 h) suffice for the elimination of most of the drug from the blood. Because the pharmacological data were obtained following oral and intraperitoneal administrations, it is reasonable to assume that the positive extended pharmacologic outcomes depend on the mode of administration. This necessitated the examination of the pharmacokinetics of *cis*-ACCP following these modes of administration.

The physicochemical properties of *cis*-ACCP predict poor permeability through biological membranes unless active transport plays a role. Indeed, comparison of the pharmacokinetic results of the per-oral administration relative to the intravenous concentration−time data show that the oral bioavailability of *cis*-ACCP is quite low, ~0.3%. The same PK profile reveals a prolonged absorption phase that occurs not only in the small intestine, but also in the colon. Thus, the process of oral absorption is extended in a manner that usually is seen in

controlled release delivery systems. This pharmacokinetic profile can explain the pharmacodynamic efficacy found following once a day oral dosing of *cis*-ACCP.

Analysis of the urine excretion of *cis*-ACCP highlights the fact that following intravenous administration the drug is excreted largely (83.9%) intact (Table 3). In contrast, only 48% of the dose administrated intraperitoneally was detected in the urine collected in 48 h. This shows that about half of the *cis*-ACCP dose is still available at the administration site and indicates a very slow release of the drug from the peritoneal cavity (Figures 8 and 9). This is especially accentuated by the finding that, during 24 h, less than 12% of the administered dose was recovered in the blood (Table 5). A similar phenomenon has been found following oral administration, where the amount of *cis*-ACCP recovered in the urine after 48 h was 6-fold greater than the amount found in the blood during the first 6 h (Table 3). Taken together, the urine pharmacokinetic data emphasize the prolonged and sustained absorption pattern of *cis*-ACCP following the different modes of administration.

To corroborate the absorption data, we have tested the permeability of *cis*-ACCP through viable pieces of intestine taken from different regions of the gut of rats in Ussing diffusion chambers. Indeed, the results had shown a pattern of low but equal degree of permeability all along the intestine, including the colon. The similarity between the permeability coefficients found for *cis*-ACCP and both atenolol and mannitol seems to indicate that it is absorbed mainly by a paracellular mechanism. This data also clarifies that there is no active component in the intestinal absorption mechanism of this compound. The slow and sustained absorption explains the *flip-flop* pharmacokinetics that characterizes *cis*-ACCP. The low rate of penetration of *cis*-ACCP through the intestine wall leads to plasma concentrations, which are significantly higher than the MIC (~12 ng/ml) for a prolonged time following oral administration.

These results can also explain the dose dependence of the pharmacological activity of *cis*-ACCP observed in the in vivo tests. A larger dose afforded better antimetastatic activity due to longer duration of effective MMP inhibitor concentration in the blood and in the extracellular fluids. Similar intestinal permeability data were found in both rats and mice. The bridging pharmacokinetic experiment done in mice confirmed the similarity in the pharmacokinetic process of *cis*-ACCP in the two species and allowed integration of the pharmacokinetic data found in rats into the pharmacokinetic−pharmacodynamic data of mice, in which the actual pharmacological studies took place.

The intraperitoneal injections are usually being used in preclinical evaluation due to convenience of administration, and there is a general, unwritten notion that it differs only slightly from the intravenous route. In the case of *cis*-ACCP, however, the results obtained from the intraperitoneal route of administration were very different from those obtained from direct administration into the systemic blood circulation. The data show that the *cis*-ACCP plasma levels versus time are higher than the MIC values for a prolonged time (~24 h), indicating slow absorption of the drug from this site of administration. The same pattern of pharmacokinetic profile was recorded following intraperitoneal administration to mice, which can explain the efficacy of the single daily dose of intraperitoneal *cis*-ACCP treatment.

Thus, *cis*-ACCP is a novel MMP inhibitor characterized by rapid systemic elimination. Its limited permeability through biological membranes enforces slow and sustained absorption to the systemic circulation enabling it to maintain effective concentration in the extracellular fluids, where it exerts its antimetastatic activity, as the active compound does not permeate into cells to induce adverse effects. Even with its poor oral bioavailability, *cis*-ACCP was found to have a sufficiently high quasi steady state concentration in the extracellular fluid, significantly above the observed MIC, for a prolonged period of time that enables once a day administration.

In conclusion, *cis*-ACCP is a medium potency, water soluble MMP inhibitor, which because of its ideal pharmacological, toxicological, and pharmacokinetic profiles appears preferable over previous, much more potent MMP inhibitors that have reached phase 2 and phase 3 clinical trials in recent years and have failed.[17–19]

Experimental Section

Chemicals. Unless specified, all reagents and chemicals have been purchased from Sigma-Aldrich, St. Louis, MO. HPLC grade water, methanol, and acetonitrile have been purchased from J.T. Baker (Holland). Recombinant matrix metalloproteinases have been purchased from R&D Systems, Minneapolis.

Animals. Mice. C57Bl mice aging 3–4 weeks, weighing 20–25 g, were used in all experiments where mice are indicated.

Rats. Male Wistar rats weighing 250–300 g were used for the ex vivo permeability study and in vivo evaluation of the compound pharmacokinetics.

The project adhered to the principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985). All animals used in the pharmacokinetic studies were deprived of food but not water 12 h prior to the experiments and during the first 12 h of the experiment. The organic synthetic procedures can be found on pages S2−S4 in the Supporting Information.

In Vitro Assays. Basement Membrane Invasiveness. Boyden chamber chemo-invasion assays were performed as previously described. Matrigel (25 ug) 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 chemo-attractant. HT-1080 human fibrosarcoma 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 (200k 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 have traversed the Matrigel layer and attached to the lower surface of the filter were stained with Diff Quick (American Scientific Products) and counted.

Chemotaxis. Chemotaxis evaluation was performed in a similar way to basement membrane invasion, with the exception that the filters are coated with 5 μg collagen IV instead of Matrigel. This amount of collagen does not form a barrier to the migrating cells, but rather an attachment substratum.

Determination of MMP Inhibitory Potency. Recombinant enzymes, human MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13, and TACE (R&D Systems, Minneapolis) were incubated at four different concentrations with the relevant colorimetric or fluorescent peptide substrates (R&D Systems, Minneapolis) for 3 h. The examined compounds were added at four to six different concentrations, and the inhibitory potencies expressed in a colorimetric change were measured by an ELISA or fluorescent reader.

In Vivo Assays. Murine Melanoma Model. Experimental metastasis was studied in the murine melanoma model. In this model, B16F10 tumor cells (50k) were injected into the tail vein of C57Bl six-week-old female mice. After 21 days, the metastases formed on the lungs of the mice were counted after appropriate fixation. Three groups of eight mice were used in this study. Two groups of eight mice were treated with daily (except weekends) administration of 50 mg/kg of ACCP. One group received the compound intraperitoneally, and the other group received the compound orally, dissolved in PBS. Mice were monitored for toxic symptoms.

Orthotopic Model of Human Prostate Tumor. In a second experimental model, we used an orthotopic model of human prostate

tumor in severe combined immunodeficiency (SCID). In this model, the tumor cells were transfected with the luciferase gene as a reporter marker. Tumor growth and tumor dissemination was followed weekly with a CCCD camera using luciferin as a detecting device.[20] The mice were treated for six weeks by daily intraperitoneal injections, except weekends, of 50 mg/kg of the tested drug.

Pharmacokinetic Experiments. Diffusion in Isolated Rat Intestine (Ussing). *cis*-ACCP was dissolved in rat mucosa buffer to create a solution with the concentration of 600 μg/mL, and 3 mL of this solution was inserted into the mucosal side of Ussing diffusion chambers containing different intestine segments of the same rat. Four Wistar rats were sacrificed and intestine segments of the jejunum, ileum, and column were assembled in the Ussing chambers to determine the diffusion of *cis*-ACCP. Samples of 100 μL were withdrawn at time 0 and 150 min from the mucosal side and at times 30, 60, 90, 120, and 150 min from the serosal side and stored immediately at −20 °C until the analysis. The withdrawn amounts of the solutions from the Ussing chambers were replaced by fresh mucosa buffer.

Pharmacokinetic Evaluation of *cis*-ACCP in Rats and Mice. Intravenous, Oral, and Intraperitoneal Administration of *cis*-ACCP to Rats. *cis*-ACCP was administered intravenously (50 mg/kg), orally (150 mg/kg), or intraperitoneally (150 mg/kg) to mice weighing 320–370 g. The compound was dissolved in normal saline/water (60/40; solution concentration: 20 mg/mL).

Following intravenous injection, blood samples of 250 μL were withdrawn at time points 5, 10, 20, 30, 60, 90, 120, 180, and 240 min. Following oral and intraperitoneal administration, blood sampling was extended to 8 and 24 h, respectively. The blood was centrifuged at 5000 rpm for 5 min, and 100 μL of the supernatant plasma was transferred to Eppendorf tubes and stored at −20 °C until being analyzed. Rats were kept in metabolic cages, and urine samples were collected 24 and 48 h after the start of each experiment and stored at −20 °C for the evaluation of urinal excretion of *cis*-ACCP.

Intraperitoneal and Oral Administration of *cis*-ACCP to Mice. *cis*-ACCP was administered intraperitoneally (50 mg/kg) or orally (150 mg/kg) to C57 mice weighing 22–25 g, which were deprived of food for 12 h before the experiment. The compound was dissolved in normal saline/water (60/40; solution concentration: 20 mg/mL). The mice were anesthetized by ether, then blood samples of 500 μL were withdrawn from the portal vein at time points 0, 30, 60, 120, 240, 360, and 720 min (one mouse was used for each sample and was), and then sacrificed by spinal dislocation after sampling. The blood was centrifuged at 5000 rpm for 5 min and 200 μL of the supernatant plasma was transferred to Eppendorf tubes and stored at −20 °C until being analyzed.

Analysis. Sample Preparation. The frozen samples (from the Ussing chambers, urine, and plasma) were allowed to warm to room temperature, and 10 μL of acyclovir solution (20 μg/mL in water) was added as internal standard. After gentle vortex, 200 μL of acetonitrile was added, and the samples were vigorously vortexed for one minute, centrifuged at 7000 rpm for 10 min, and 230 μL of the clear supernatant was transferred to glass tubes to be subjected to vacuum evaporation at room temperature to dryness. The dry residue was partitioned between 150 μL of 0.1 N HCl solution and 0.5 mL of ethyl acetate, vigorously vortexed for one minute, and centrifuged at 5000 rpm for 5 min.

A total of 100 μL of the aqueous lower fraction was transferred to analysis tubes and analyzed by HPLC-MS against a freshly prepared calibration curve.

Calibration curves have been prepared on the day prior to the analysis. Samples of plasma containing the following concentrations of *cis*-ACCP were prepared by spiking the compound in fresh blank plasma: 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 75, 100, 200, and 400 μg/mL. To prepare a calibration curve in the urine, concentrations of 10, 25, 50, 100, 200, 500, and 1000 μg/mL of *cis*-ACCP in blank urine were prepared. The samples were then treated as mentioned above.

Instrument. HPLC-MS: Waters Millennium HPLC-MS instrument equipped with Micromass ZQ detector, Waters 600 Controller gradient pump, and Waters 717 autosampler.

Column. Waters Xterra MS C_{18} column 2.1 × 150 mm was used with the following settings: nitrogen flow, 500 L/h; desolvation temp, 400 °C; source temp., 150 °C; cone voltage, 15 V.

Mobile Phase. The mobile phase consisted of 3% methanol, 97% water, 0.1% formic acid, and 0.05% trifluoroacetic acid.

Flow rate. A flow gradient was applied as follows: 1–3 min, 0.1 mL/min; 3–3.5 min, flow increase to 0.2 mL/min; 3.5–12 min, 0.2 mL/min; 12–13 min, flow rate decrease to 0.1 mL/min; and 13–15 min, 0.1 mL/min. No change in the mobile phase constituent was done during the analysis.

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Supporting Information Available: The organic synthetic procedures, analytical data for the final compounds and some intermediates, and graphs of the time dependence and mode of inhibition by *cis*-ACCP are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Jacobsen, F. E.; Lewis, J. A.; Cohen, S. M. The design of inhibitors for medicinally relevant metalloproteins. *ChemMedChem.* **2007**, *2*, 152–171. (b) Hu, J.; Van den Steen, P. E.; Sang, Q.-X. A.; Opdenakker, G. Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat. Rev. Drug Discovery* **2007**, *6*, 480. (c) Fisher, J. F.; Mobashery, S. Recent advances in MMP inhibitor design. *Cancer Metastasis Rev.* **2006**, *25*, 115–136. (d) Sang, Q.-X. A.; Jin, Y.; Newcomer, R. G.; Monroe, S. C.; Fang, X.; Hurst, D. R.; Lee, S.; Cao, Q.; Schwartz, M. A. Matrix metalloproteinase inhibitors as prospective agents for the prevention and treatment of cardiovascular and neoplastic diseases. *Curr. Top. Med. Chem.* **2006**, *6*, 289–316. (e) Rao, B. G. Recent developments in the design of specific matrix metalloproteinase inhibitors aided by structural and computational studies. *Curr. Pharm. Des.* **2005**, *11*, 295–322. (f) Skiles, J. W.; Gonnella, N. C.; Jeng, A. Y. The design, structure, and clinical update of small molecular weight matrix metalloproteinase inhibitors. *Curr. Med. Chem.* **2004**, *11*, 2911–2977. (g) Doherty, T. M.; Asotra, K.; Pei, D. Q.; Uzui, H.; Wilkin, D. J.; Shah, P. K.; Rajavashisth, T. B. Therapeutic developments in matrix metalloproteinase inhibition. *Expert Opin. Ther. Pat.* **2002**, *12*, 665–707. (h) Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H. Design and therapeutic application of matrix metalloproteinase inhibitors. *Chem. Rev.* **1999**, *99*, 2735–2776.

(2) Ramamurthy, N. S.; Rifkin, B. R.; Greenwald, R. A.; Xu, J. W.; Liu, Y.; Turner, G.; Golub, L. M.; Vernillo, A. T. Inhibition of matrix metalloproteinase-mediated periodontal bone loss in rats: A comparison of six chemically modified tetracyclines. *J. Periodontol.* **2002**, *73*, 726–734.

(3) (a) Breuer, E.; Salomon, C. J.; Katz, Y.; Chen, W.; Lu, S.; Röschenthaler, G.-V.; Hadar, R.; Reich, R. Carbamoylphosphonates—A new class of in vivo active matrix metalloproteinase inhibitors 1. Alkyl- and cycloalkylcarbamoylphosphonic acids. *J. Med. Chem.* **2004**, *47*, 2826–2832. (b) Breuer, E.; Katz, Y.; Hadar, R.; Reich, R. Carbamoylphosphonate MMP inhibitors 4. The influence of chirality and geometrical isomerism on the potency and selectivity of inhibition. *Tetrahedron: Asymmetry* **2004**, *15*, 2415–2420.

(4) Reich, R.; Katz, Y.; Hadar, R.; Breuer, E. Carbamoylphosphonate MMP inhibitors 3. In vivo evaluation of cyclopentylcarbamoylphosphonic acid, CPCPA, in experimental metastasis and angiogenesis. *Clin. Cancer Res.* **2005**, *11*, 3925–3929.

(5) Farkas, E.; Katz, Y.; Bhusare, S.; Reich, R.; Röschenthaler, G. V. M.; Königsmann, M.; Breuer, E. Carbamoylphosphonate based matrix metalloproteinase (MMP) inhibitor metal complexes—Solution studies and stability constants towards a zinc selective binding group. *J. Biol. Inorg. Chem.* **2004**, *9*, 307–315.

(6) It should be mentioned that both ACCP studied are racemic mixtures. Our attempts to synthesize an enantiomerically pure *cis*-isomer, so

far, did not succeed. The starting material, *cis*-1,2-diaminocyclohexane, is a meso compound, therefore, a synthesis cannot be based on an optically active starting material. We have attempted an enantioselective synthesis of one of the enantiomers of *cis*-ACCP starting from cyclohexene oxide, using the methodology of Govindaraju et al., as follows: Govindaraju, T.; Gonnade, R. G.; Bhadbhade, M. M.; Kumar, V. A.; Ganesh, K. N. 1 *S*,2*R*/1*R*,2*S*)-Aminocyclohexyl glycyl thymine PNA: Synthesis, monomer crystal structures, and DNA/RNA hybridization studies. *Org. Lett.* **2003**, *5*, 3013–3016, however, we could not avoid racemization in the next to the last step in the synthesis. In case of *trans*-isomer, it should be possible to synthesize both enantiomers of *trans*-ACCP starting from the commercially available (*R*,*R*) and (*S*,*S*)-diaminocyclohexanes, but in view of the weak biological activity of racemic *trans*-ACCP, this is of low priority for us at this time.

(7) Goldblum, A. and Shehadeh, C., private communication.

(8) For example, one compound has been found to possess IC_{50} values of 20 μM or 150 μM when determined on MMP-2 from two different sources. See references 9a and 9b.

(9) (a) Paemen, L.; Martens, E.; Norga, K.; Masure, S.; Roets, E.; Hoogmartens, J.; Opdenakker, G. The gelatinase inhibitory activity of tetracyclines and chemically modified tetracycline analogues measured by a novel microtiter assay for inhibitors. *Biochem. Pharmacol.* **1996**, *52*, 105–111. (b) Seftor, R. E. B.; Seftor, E. A.; De Larco, J. E.; Kleiner, D. E.; Leferson, J.; Stetler-Stevenson, W. G.; McNamara, T. F.; Golub, L. M.; Hendrix, M. J. C. Chemically modified tetracyclines inhibit human melanoma cell invasion and metastasis. *Clin. Exp. Metastasis* **1998**, *16*, 217–225.

(10) Reich, R.; Thompson, E. W.; Iwamoto, Y.; Martin, G. R.; Deason, J. R.; Fuller, G. C.; Miskin, R. Inhibition of plasminogen activator, serine proteinases, and collagenase IV prevents the invasion of basement membrane by metastatic cells. *Cancer Res.* **1988**, *48*, 3307–3312.

(11) Talmadge, J. E.; Singh, R. K.; Fidler, I. J.; Raz, A. Murine models to evaluate novel and conventional therapeutic strategies for cancer. *Am. J. Pathol.* **2007**, *170*, 793–804.

(12) Brandstetter, H.; Grams, F.; Glitz, D.; Lang, A.; Huber, R.; Bode, W.; Krell, H.-W.; Engh, R. A. The 1.8 Å crystal structure of a matrix metalloproteinase 8-barbiturate inhibitor complex reveals a previously unobserved mechanism for collagenase substrate recognition. *J. Biol. Chem.* **2001**, *276*, 17405–17412.

(13) Breuer, E.; Frant, J.; Reich, R. Recent nonhydroxamate matrix metalloproteinase inhibitors. *Expert Opin. Ther. Pat.* **2005**, *15*, 253–269.

(14) O'Brien, E. C.; Farkas, E.; Gil, M. J.; Fitzgerald, D.; Castineras, A.; Nolan, K. B. Metal complexes of salicylhydroxamic acid (H_2Sha), anthranilic hydroxamic acid and benzohydroxamic acid. Crystal and molecular structure of [Cu(phen)2(Cl)]Cl × H2Sha, a model for a peroxidase−inhibitor complex. *J. Inorg. Biochem.* **2000**, *79*, 47–51.

(15) McNabb, J.; Bui, K. Q. β-Lactam Pharmacodynamics. In *Antimicrobial Pharmacodynamics in Theory and Clinical Practice*; Nightingale, C. H., Murakawa, T., Ambrose, P. G.; Eds.; Marcel Dekker, Inc.: New York, 2002; pp 99–124.

(16) Hoffman, A.; Danenberg, H. D.; Katzhendler, I.; Shuval, R.; Gilhar, D.; Friedman, M. Pharmacodynamic and pharmacokinetic rationales for the development of an oral controlled-release amoxicillin dosage form. *J. Controlled Release* **1998**, *54*, 29–37.

(17) *cis*-ACCP is far more potent than the tetracycline-derived MMP inhibitors (IC_{50} values reported to be in the range of 20–500 μM[9]) that are currently limited to periodontal use but are under investigation in connection with other clinical indications.[18,19]

(18) (a) Roach, D. M.; Fitridge, R. A.; Laws, P. E.; Millard, S. H.; Varelias, A.; Cowled, P. A. Up-regulation of MMP-2 and MMP-9 leads to degradation of type IV collagen during skeletal muscle reperfusion injury: Protection by the MMP inhibitor, doxycycline. *Eur. J. Vasc., Endovasc. Surg.* **2002**, *23*, 260–269. (b) Rudek, M. A.; Figg, W. D.; Dyer, V.; Dahut, W.; Turner, M. L.; Steinberg, S. M.; Liewehr, D. J.; Kohler, D. R.; Pluda, J. M.; Reed, E. Phase I clinical trial of oral COL-3, a matrix metalloproteinase inhibitor, in patients with refractory metastatic. *Cancer J. Clin. Oncol.* **2001**, *19*, 584–592. (c) Chu, Q. S. C.; Forouzesh, B.; Syed, S.; Mita, M.; Schwartz, G.; Copper, J.; Curtright, J.; Rowinsky, E. K. A phase II and pharmacological study of the matrix metalloproteinase inhibitor (MMPI) COL-3 in patients with advanced soft tissue sarcomas. *Invest. New Drugs* **2007**, *25*, 359–367.

(19) A somewhat similar opinion has been advanced recently by the developers of SAHA, an inhibitor of histone deacetylase and also a zinc enzyme. SAHA (suberoylanilide hydroxamic acid; vorinostat, Zolinza) is an inhibitor of medium potency, which has recently won the approval of the FDA. It has been pointed out by these authors that vorinostat was better than its numerous higher potency analogs, which had unacceptable side effects and, therefore, had to be discontinued: Marks, P. A.; Breslow, R. Dimethyl sulfoxide to vorinostat: Development of this histone deacetylase inhibitor as an anticancer drug. *Nat. Biotechnol.* **2007**, *25*, 84–90.

(20) Honigman, A.; Zeira, E.; Ohana., P.; Abramovitz, R.; Tavor, E.; Bar, I.; Zilberman, Y.; Rabinovsky, R.; Gazit, D.; Joseph., A.; Panet, A.; Shai, E.; Palmon, A.; Laster, M.; Galun, E. Imaging transgene expression in live animals. *Mol. Ther.* **2001**, *4*, 239–249.

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