Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Carbamoylphosphonates Control Tumor Cell Proliferation and Dissemination by Simultaneously Inhibiting Carbonic Anhydrase IX and Matrix Metalloproteinase-2. Toward Nontoxic Chemotherapy Targeting Tumor Microenvironment[1]

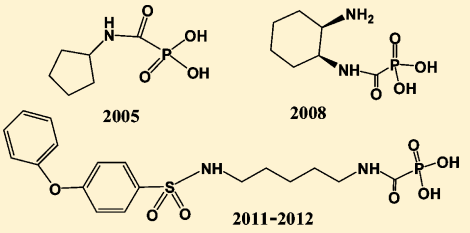
Reuven Reich,[‡] Amnon Hoffman,[‡] Ainelly Veerendhar,[‡] Alfonso Maresca,[§] Alessio Innocenti,[§] Claudiu T. Supuran,*[,§] and Eli Breuer*[,‡]

[‡]Institute for Drug Research, The Hebrew University of Jerusalem, School of Pharmacy, P.O. Box 12065, Jerusalem IL 91120, Israel

[§]Universita degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Florence), Italy

Supporting Information

ABSTRACT: Carbamoylphosphonates (CPOs) have been identified as inhibitors of matrix metalloproteinases (MMPs) and as orally active, bioavailable, and safe antimetastatic agents. In this article, we focus on the direct antitumor activity of the CPOs. We discovered that CPOs also inhibit carbonic anhydrases (CAs), especially the IX and XII isoforms identified as cancer promoting factors. Thus, CPOs can be regarded as novel nontoxic drug candidates for tumor microenvironment targeted chemotherapy acting by two synergistic mechanisms, namely, inhibiting CAs and MMPs simultaneously. We have also demonstrated that the ionized CPO acid is unable to cross the cell membrane and thus limited to interact with the extracellular domains of isozymes CAIX and CAXII. Finally, applying CPOs against cancer cells in hypoxic conditions resulted in the dose dependent release of lactate dehydrogenase, confirming the direct interaction of the CPOs with the cancer related isozymes CAIX and XII and thereby promoting cellular damage.



INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of about 26 zinc endopeptidases, which collectively have the capacity to degrade all the major components of the extracellular matrix and have crucial roles in inflammation, cancer dissemination, and other indications.[2] Although the MMPs play a crucial role in physiological tissue remodeling and repair, their overexpression has been linked with a variety of chronic diseases including cancer, arthritis,[3] osteoporosis,[4] multiple sclerosis,[5] arteriosclerosis,[6] restenosis,[5] meningitis,[7] congestive heart failure,[8] chronic obstructive pulmonary disease,[9] chronic wounds[7a], liver cirrhosis,[10] cerebral ischemia,[11] and others. After more than twenty years of worldwide research, significant advances have been made in the clarification of the mechanism of inhibition, yet no clinically useful inhibitor has been identified. The main reason for the failure of these trials to reach their end-points was that severe painful side effects, e.g., the musculoskeletal syndrome, necessitated interruption of the clinical trials. Other reasons were that, while some MMPs promote pathology, others have a protective effect.[12] Also, previous attempts to consider MMPs as targets were burdened by the lack of information on which MMP does what and where in physiology and pathology. Nowadays, we have a much better understanding of the individual roles of the various orthologs.[13,14] In addition, all first generation compounds—and most of the second generation ones—were hampered by lack of specificity, i.e., they targeted all MMPs and mostly also ADAMs. Consequently, current efforts are focused on finding selective inhibitors, those that do not bind zinc at all,[15,16] or phosphonates.[17,18]

Our work has been focused on metastasis formation in which MMPs (especially MMP-2) have a vital role by mediating tumor cell dissemination. Efficient inhibition of MMPs is, therefore, an important therapeutic target, which has attracted considerable attention within academia and industry for the last two decades or so.[19,20]

In recent years, we have reported in a series of papers that carbamoylphosphonates (CPOs) possess zinc binding ability[21] and are able to act both in vitro and in vivo as inhibitors of MMPs.[22−26]

In this article, we wish to report our discovery that the above-mentioned CPOs also inhibit carbonic anhydrases (CAs), a family of about 16 enzymes that catalyze the interconversion of carbon dioxide and bicarbonate to maintain the required pH in biological fluids. CAs are involved in many physiological and pathological processes, including respiration and transport of CO_2 and bicarbonate between metabolizing tissues and lungs; pH and CO_2 homeostasis; electrolyte secretion in various tissues and organs; biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis); bone resorption; calcification; and

Received: July 7, 2012
Published: August 15, 2012

tumorigenicity.[27] Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited to treat a range of disorders including edema, glaucoma, obesity, cancer, epilepsy, and osteoporosis.[28]

In mammals, 16 α-CA isozymes or CA-related proteins have been described with different catalytic activity, subcellular localization, and tissue distribution. There are five cytosolic forms (CA I, CA II, CA III, CA VII, and CA XIII), five membrane bound isozymes (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial forms (CA VA and CA VB), and a secreted CA isozyme (CA VI).

In the present article, we report inhibition constants of 21 CPOs on four CAs: CAI, CAII, CAIX, and CAXII. The latter two isozymes, CAIX and XII, are membrane-type isoforms with *extracellularly exposed* active sites, which are induced by the transcription factor, Hypoxia Induced Factor 1α (HIF-1α).[29] They are present in the membranes of tumor cells and are critical for their proliferation. Because they catalyze the reversible hydration of carbon dioxide leading to bicarbonate and proton formation, their upregulation results in decrease of extracellular pH, and it is strongly associated with cancer cell survival and malignant progression.[30,31] Using various breast cancer cell lines,[32] it has been recently demonstrated that CAIX is essential to the survival of tumor cells under hypoxic condition of breast tumor, and its activity contributes to metastasis generation, and could serve as a specific biomarker for this kind of tumor. The necessity of CAIX to tumor and metastasis formation has also been recently demonstrated by using knock-down approach.[33] Further, inhibition of CAIX has been shown to result in regression or growth inhibition of mouse and human breast tumors as well as inhibition of cancer metastasis formation.[32]

Both CAs and the MMPs are zinc enzymes that reside and function in the extracellular environment; therefore, it is not surprising that they are inhibited by the same zinc-binding, ionic, and water-soluble inhibitors. From this, it follows that the anticancer and antimetastatic properties that we have observed over the last eight years in the in vivo examination of CPOs, as reported in our previous papers,[22,23,25,26] have originated from the simultaneous inhibition of both CA and MMPs enzymatic systems.

Therefore, it was of interest to examine in detail CPOs (aka phosphonoformamides) as CA inhibitors. The results of these studies are described in this paper.

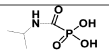
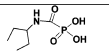
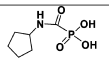
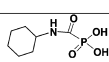
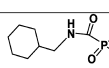
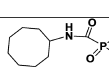
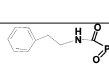
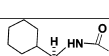
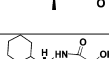
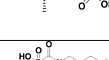
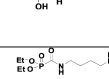
RESULTS

Carbamoylphosphonates (CPOs) Inhibit Carbonic Anhydrases. The CA inhibition constants of representative CPOs on four isozymes, human carbonic anhydrases I, II, IX, and XII (hCAI, hCAII, hCAIX, and hCAXII) are reported in three tables. Beside the CA inhibition constants, the CPOs' MMP-2 inhibition constants have also been added in a separate column.

The results for aliphatic and alicyclic compounds are listed in Table 1. It is evident from this table that there is no structure–activity relationship apparent in the obtained results. All IC_{50} values are of the same order of magnitude, whether they are chain compounds (**1** and **2**) or ring compounds (**3**–**7**). Neither the aliphatic nor aromatic nature of the rings has any significant effect (compare **3**–**6** vs **7**). Furthermore, the pair of enantiomers, **8** and **9**, also show similar inhibition constants, in contrast to their differing inhibition constants on MMPs.[24] Finally, Table 1 also contains 1,4-butanebiscarbamoylphosphonic acid and its tetraethyl ester (compounds **10** and **11**, respectively),[34] both of which show similar inhibition characteristics to the mono-CPO compounds mentioned above. Among the compounds in Table 1, an in-depth study was devoted to cyclopentyl-CPO (**3**, CPCPA),[23] which revealed significantly inhibited cellular invasion and capillary formation in vitro. Further, both i.p. or oral administration of the compound significantly reduced lung metastasis formation and s.c. tumor growth in a murine melanoma model.

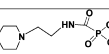
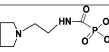
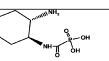
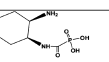
Table 2 shows aminoalkyl- and aminocycloalkyl-CPOs. Earlier, we reported that the two acyclic aminoalkylCPOs **12** and **13**, as well as the alicyclic cis-ACCP (**15**), were able to reduce TNFα secretion to a level equivalent to the reduction caused by the steroid drug, budesonide. The reduction in TNFα levels was

Table 1. Inhibition Constants of Alkyl- and Cycloalkyl-CPOs on MMP2, hCAI, hCAII, hCAIX, and hCAXII[a]

#	Symbol	Structure	MMP-2 $IC_{50}\mu M$ (ref)	hCAI $IC_{50}\mu M$	hCAII $IC_{50}\mu M$	hCAIX $IC_{50}\mu M$	hCAXII $IC_{50}\mu M$
1	YK-102		1 (22)	8.81	13.87	8.30	7.27
2	YK-137		3 (22)	6.71	8.79	8.26	4.65
3	YK-96		0.08 (22)	8.79	7.41	8.97	7.26
4	YK-83		3 (22)	7.89	8.81	8.89	5.59
5	YK-103		0.2 (22)	7.36	9.23	8.59	7.44
6	YK-111		1 (22)	8.02	7.89	9.14	7.99
7	YK-9		20 (22)	7.68	8.00	6.08	5.44
8	(*S*) YK-145		1 (24)	5.31	9.45	6.36	6.64
9	(*R*) YK-146		0.2 (24)	6.78	8.85	8.12	6.97
10	Bin-19		n.d. (34)	9.74	8.82	9.10	6.02
11	Bin-3		n.d. (34)	9.70	9.59	9.65	2.72

[a]n.d. = not determined.

Table 2. Inhibition Constants of Aminoalkyl- and AminocycloalkylCPOs on MMP-2, hCAI, hCAII, hCAIX, and hCAXII

Cpd. No.	Symbol	Structure	MMP-2 $IC_{50}\mu M$ (ref)	hCAI $IC_{50}\mu M$	hCAII $IC_{50}\mu M$	hCAIX $IC_{50}\mu M$	hCAXII $IC_{50}\mu M$
12	Ch-109 PIPCPO		4 (35)	7.82	8.60	9.00	7.64
13	Ch-112 PYRCPO		4 (35)	9.43	9.66	8.80	5.43
14	YK-154 trans-ACCP		>100 (25)	8.24	8.28	7.14	8.26
15	YK-162 cis-ACCP		4 (25)	1.6	1.32	8.60	6.00

dx.doi.org/10.1021/jm300981b | J. Med. Chem. 2012, 55, 7875–7882

Table 3. Inhibition Constants of Arylsulfonamido- and CarboxamidoCPOs onMMP-2, hCAI, hCAII, hCAIX, and hCAXII[a]

#	symbol	structure	MMP-2 $IC_{50}\mu M$	IC_{50} μM hCAI	IC_{50} μM hCAII	IC_{50} μM hCAIX	IC_{50} μM hCAXII
16	JS-268	4-$PhOC_6H_4SO_2NH(CH_2)_2NHCOPO_3H_2$	15	0.98	0.82	7.0	6.9
17	JS-325	4-$PhOC_6H_4SO_2NH(CH_2)_3NHCOPO_3H_2$	10	0.71	0.58	6.8	6.4
18	JS-343	4-$PhOC_6H_4SO_2NH(CH_2)_4NHCOPO_3H_2$	7	0.75	0.1	6.1	5.6
19	JS-389	4-$PhOC_6H_4SO_2NH(CH_2)_5NHCOPO_3H_2$	4	0.52	0.31	6.3	5.7
20	JS-403	4-$PhOC_6H_4SO_2NH(CH_2)_6NHCOPO_3H_2$	4	0.38	0.10	7.0	6.2
21	JS-294	4-$PhOC_6H_4CONH(CH_2)_2NHCOPO_3H_2$	20	0.62	0.61	9.9	8.6

[a]Compounds **16**–**20** have been reported in ref 26. Compound **21** has been reported in ref 36.

Table 4. In Vivo Results of Dual MMP-2 and CA Inhibitors

CPO #	symbol	drug dose mg/kg	model	mode of drug introduction	% decrease in (lung) metast, of control	ref
3	YK-96 CPCPA	50	Melanoma	IP	86	22
3	YK-96 CPCPA	50	Melanoma	Oral	80	23
3	YK-96 CPCPA	50	Subcutan. Tumor	IP	??	23
15	YK-162 cis-ACCP	50	Melanoma	IP	70	25
15	YK-162 cis-ACCP	50	Melanoma	Oral	95	25
15	YK-162 cis-ACCP	50	Orthotopic H. Prostate	IP	~90	25
19	JS-389	12.5	Melanoma	IP	57	26
19	JS-389	25	Melanoma	IP	76	26
19	JS-389	50	Melanoma	IP	90	26
19	JS-389	50	Melanoma	oral	80	26
20	JS-403	12.5	Melanoma	IP	82	26
20	JS-403	25	Melanoma	IP	90	26
20	JS-403	50	Melanoma	Oral	50	26

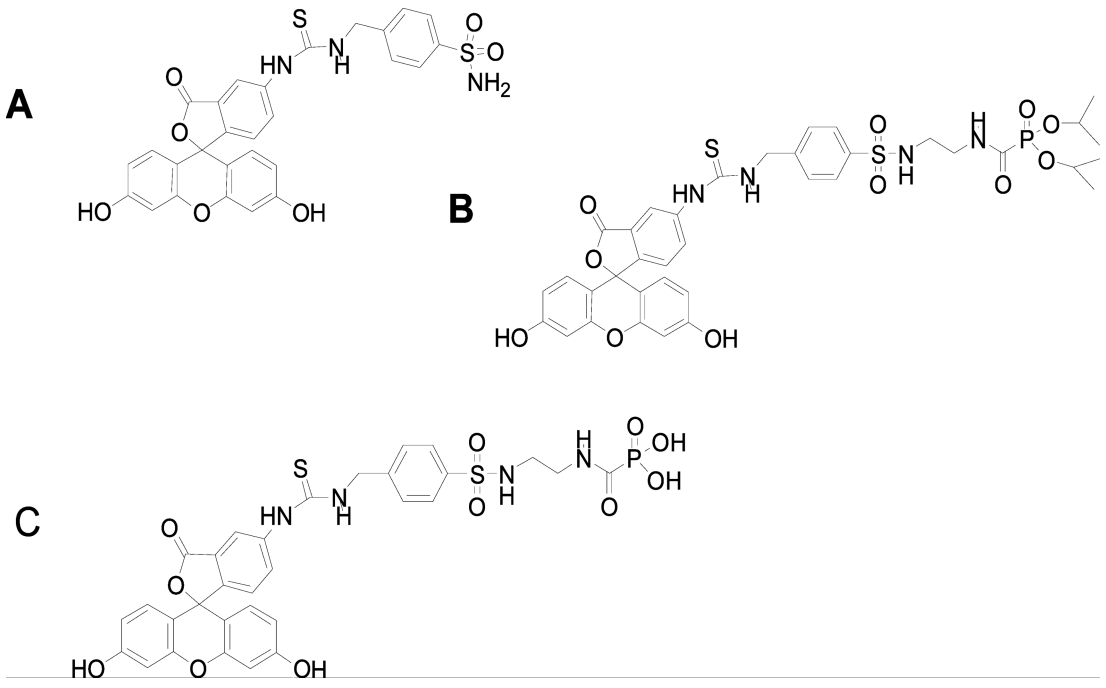


Figure 1. Structures of the three fluorescent probes used in this work.

not accompanied by cytotoxicity, nor did it inhibit cell proliferation.[35] Among the compounds in this table, we singled out **15** (cis-ACCP) for an in-depth examination, which included two in vivo models and a pharmacokinetic study.[25] Compound **15** (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 **15**, cis-ACCP, in rats revealed distribution restricted into the extracellular fluid, which is the site of action for the antimetastatic activity.[25]

Finally, Table 3 lists a series of five 4-phenoxybenzenesulfonamidoalkylCPOs[11] and one 4-phenoxybenzamidoalkylCPO, compound **21** (JS-294). These sulfonamides, having varying lengths of polymethylene chains, have been evaluated previously as MMP inhibitors and showed gradual improvement in their MMP-2 inhibitory ability with the lengthening of the chain. In Table 3, we see, in contrast, that the CA inhibitory ability does

dx.doi.org/10.1021/jm300981b | J. Med. Chem. 2012, 55, 7875–7882

not depend on the chain length at all. While the compounds listed in Tables 1 and 2 have only carbamoylphosphonates as zinc-binding-groups, in Table 3 we see, for the first time, molecules having sulfonamide groups near the carbamoylphosphonic groups. Yet, the addition of the latter does not lead to more potent CAIs than the CPOs lacking sulfonamides. Furthermore, comparing compounds **16** and **21** (JS-294), which have the same methylene chain lengths, we see that the replacement of the sulfonamide group of **16** by a carboxamide in **21**[36] keeps the inhibition profiles in the same order of magnitude indicating that carbamoylphosphonates are potent CAIs in their own right, without requiring contribution of sulfonamides.

Table 4 contains a summary of the in vivo results obtained from tested CPOs mentioned earlier and originally were attributed to MMP inhibition. This view now needs to be modified with the inclusion of the effects of CAIX and CAXII. The last column contains references to the papers containing the original in vivo results.

Membrane Impermeability of Carbamoylphosphonates. The question of carbamoylphosphonate anions' cell membrane permeability is one of the critical issues regarding the scope of action of these inhibitors. Inhibitors that are able to penetrate into the cells will inhibit the cytosolic CA I, II, III, VII, and XIII and the mitochondrial CA VA and VB, while those that cannot enter cells will only inhibit the four membrane-associated isozymes CA IV, IX, XII, and XIV, the catalytic domains of which protrude to the extracellular medium. Among the latter four, the most important isoform is CAIX, which is closely relevant to tumor cell proliferation.

We sought the answer to the question of the CPO anions' cell membrane permeability by observing the behavior of fluorescein-bound inhibitor molecule **C** and to two other control molecules **A** and **B**. The structures of the three fluorescent molecules are displayed in Figure 1.

Fluorescein derivative **A** was chosen as positive control because it is known as a molecule easily permeable into cells.[37] Molecule **B** is a carbamoylphosphonic acid diisopropyl ester linked to a fluorescein molecule. Finally, molecule **C** is a fluorescein bound carbamoylphosphonic acid ($pK_{a1} < 2$ and $pK_{a2} \approx 5-5.5$), which should be doubly ionized at physiological pH. Fluorescein derivative **A** had been synthesized according to the method of Supuran and co-workers.[37] Fluorescein derivatives **B** and **C** have been synthesized in this work as described in the Supporting Information.

The membrane permeabilities of compounds **A**, **B**, and **C** have been examined on two cell lines, (1) HT-1080 and (2) MDA-231. These cells had been exposed to the three compounds for 20 and 120 min at 37 °C. Following the incubation period, the cells were washed three times with PBS and visualized by a fluorescent inverted microscope. As shown in SI Figures 2S and 3S on HT-1080 and MDA-231cells, compound **A** penetrated quite rapidly even at 20 min. Compound **B** showed a slower penetration profile and at 120 min distributed evenly inside the cells. In contrast, compound **C** accumulated pericellularly and did not penetrate the cells.

Hypoxia. Tumor cells alter their metabolism when in a hypoxic microenvironment to survive, proliferate, and metastasize. CAs catalyze the reversible hydration of carbon dioxide (CO_2) to generate a bicarbonate anion (HCO_3^-) and a proton (H^+).[38] Carbonic anhydrase isozymes CA IX and CA XII are embedded in the membranes of tumor cells that express them, maintain a pHi range compatible with cell viability and proliferation, while they contribute to the extracellular acidification of the tumor microenvironment. These enzymes confer a survival advantage to tumor cells growing in a hypoxic and acidic microenvironment.

If an effective CAIX inhibitor would interact with a tumor cell expressing CAIX in condition of hypoxia, it should block the CAIX supported tumor cell survival. Seeking evidence for establishing the direct interaction of representative CPOs with CAIX, we turned to a similar experiment under hypoxia conditions. In the first step, we tested the feasibility with two kinds of tumor cells, namely, HT-1080 and HeLa cells. Treatment of these two kinds of cells, both of which express CAIX, with 50 μM inhibitor **19** (JS-389) caused in HeLa cells a 1.3-fold increase in lactate dehydrogenase (LDH), while the same treatment induced in HT1080 cells a 2.3-fold increase, respectively, relative to control. Since the formation of LDH reflects cell damage caused by the CAI, these preliminary results indicate that HeLa cells are less sensitive than HT1080 cells. Therefore, in the second experiment we tested dose response of LDH formation only in HT1080 cells using three doses of two inhibitors **19** (JS-389) and **20** (JS-403). The results appear in Table 5. From this table, it appears that the damage to the tumor cell caused by **19** (JS-389) is larger.

Table 5. Effect of Carbamoylphosphonate CAIs on LDH Secretion under Hypoxic Conditions

HT-1080 cells			
CA inhibitor	LDH		control
conc. μM	CAI JS-389 **19**	CAI JS-403 **20**	arbitrary value
50	2.3	1.9	1
10	1.5	1.3	1
1	1	1	1

DISCUSSION

In the course of our previous work, studying carbamoylphosphonates (CPOs) as MMP inhibitors, several CPOs have shown higher potency in vivo than that could be expected from the in vitro results. This indicated that CPOs may have one or more additional targets. The in vitro tests used for CPOs were specific to MMPs, while the in vivo test involved B16F10 or other tumor cells, which potentially express additional metalloenzymes.

One in vitro test is the determination of enzyme kinetics using recombinant MMP isozymes, while the other is an in vitro invasion assay, or chemoinvasion across a reconstituted basement membrane by tumor cells, which is dependent on the presence and activity of certain MMPs, mainly MMP-2 and MMP-9.[39]

Another in vitro test used was capillary formation, which has been shown to be dependent on expression of certain MMPs. New blood vessel formation is a critical step in the expansion and in dissemination of a given tumor. This experiment has become an accepted model of angiogenesis in vitro.[40]

On the other hand, the in vivo test used most frequently in our group is the B16F10 melanoma which is relatively convenient to carry out and yields clear results in three weeks. Another aspect of B16F10 cells is that they express CA IX, which makes them a convenient platform for testing CPOs as potential anticancer CAIs.[41] An additional coincidence is that both MMPs and CAs are zinc enzymes functioning by similar mechanisms; therefore, it is not surprising that identical or similar molecules would inhibit them.

Detailed in vivo anticancer and antimetastatic results for selected carbamoylphosphonates (Table 4) have been described mainly in three publications,[23,25,26] in which the biological activities were attributed to the compounds MMP-2 inhibitory activity. This view now needs a revision following the discovery of the dual nature of CPOs and that the observed anticancer and antimetastatic activities are the results of the dual inhibition! While inhibition of MMP-2 blocks the breakdown of the basement membranes, angiogenesis, and the dissemination of metastases, inhibition of CAIX and CAXII targets hypoxia, elevates the pH in the tumor microenvironment, and obstructs the proliferation of the cancer cells.[32] These simultaneous actions are made possible by the similarities of the two kinds of enzymes. Both MMPs and CAs are zinc enzymes that function in the extracellular environment through similar mechanisms; therefore, ideally they are inhibited by water-soluble zinc binding inhibitors. The ionized compounds such as CPOs ($pK_1 \leq 2$; $pK_2 \approx 5-5.5$) fulfill this requirement. As it is demonstrated in this paper, they are unable to penetrate cells, therefore remain in the extracellular fluid, and can be expected to be nontoxic inhibitors.

Many metal binding anions, including various phosphorus acids, have also been recognized as CA isozyme inhibitors. Thus, orthophosphate anions, carbamoyl phosphate, phosphonoformate (foscarnet), and formate have been reported as weak CAIX inhibitors (K_I's in the range of 0.56−3.67 mM), whereas mono and dihydrogen phosphates are even weaker (having K_I's 3.67 and 21.1 mM, respectively).[42]

Carbamoylphosphonates, also called phosphonoformamides, are orders of magnitude more potent CA inhibitors relative to their parent phosphonoformate. This might be the result of the electron donating effect of the nitrogen lone pair in the R—NH—C═O moiety, polarizing the carbamoyl C═O group, the effect of which might even extend to the phosphorus and strengthen its interactions with electrophiles on the enzyme. The X-ray results of foscarnet bound to a CA support such an assumption. X-ray crystallography of CA inhibited by a CPO will answer this question. Interestingly, examination of our results shows that the nature, steric hindrance, chirality, and even the esterification of the phosphonic groups in an R−NH−C(O)-PO_3H_2 molecule did not appear to influence the extent of the CA inhibition. This is in sharp contrast to MMPs whose inhibition constants are sensitive to every kind of difference in the structures of the N-substituents in the N-substituted CPOs. The degree of esterification of the $-PO_3H_2$ group is also critical for MMP inhibition. Only the completely free dibasic phosphonic acids showed MMP inhibition, in contrast to CAs, which are inhibited equally by diester and by diacid alike. This phenomenon can perhaps be exploited for designing CA inhibitors for intracellular purposes after having shown that the diisopropyl ester is able to penetrate across the cell membrane.

Other dual MMP-CA inhibitors based on hydroxamates as zinc-binding groups have also been described.[43] However, the utility of these compounds is doubtful, considering the failure of hydroxamates as clinically useful MMP inhibitors. Most hydroxamate-based MMP inhibitors are hydrophobic molecules which have non-ionizable functional groups that enable them to cross lipidic cell membranes into cells, leading to musculoskeletal[44] side effects. Another shortcoming of hydroxamates is their very strong inhibition constants and nonselective binding of all transition metals in the body, especially iron, which is present in somewhat larger quantities than zinc. The 10^6- to 10^{10}-fold higher stability constants of hydroxamate−iron complexes than those containing with zinc[21,45] is also likely to be the source of some of the toxic side effects.[46] CPOs do not cause musculoskeletal side effects and are relatively weak transition metal ligands; thus, they are free of the above-mentioned toxicity. Our attempts to determine stability constants for CPO-iron(III) complexes failed due to precipitation.[21]

In recent years, weak bonds and weak interactions are being seen and discussed more and more frequently in virtually every branch of science. Biology and its branches are no exception. One can see a statement like, "for a high degree of specificity the contact or combining spots on the two particles must be multiple and weak"[47] or "a multitude of weak, or transient, biological interactions (dissociation constant: Kd > μM), either working alone or in concert, occur frequently throughout biological systems".[48]

While medicinal chemists usually strive to discover the most potent drug possible, in some cases the advantage of a weak interaction between a drug and its receptor had been recognized and preferred.[49]

The first carbamoylphosphonate MMP inhibitors we synthesized were lacking the diphenyl ether affinity groups, and they showed MMP inhibition of short duration. Our most recent inhibitors having the diphenyl ether as a putative S_1' affinity group inhibited MMPs for as long as they were monitored (3 h), indicating higher affinity to the enzymes, yet their inhibition constants remained in the single-digit μM range, similar to the magnitude of CAIX's and CAXII's inhibition constants. This appears as the ideal situation for a double (or multiple) target inhibitor in which both targets promote the same disease (cancer and metastasis) and are inhibited by a common drug. Assuming that an inhibitor would have several orders of magnitude stronger binding constant to one target than to the other, it seems reasonable that close to 100% of the drug would bind to the stronger attracting target and the benefit from having a powerful inhibitor for two targets would be lost. In summary, there is an advantage in having a relatively weakly binding drug to two or multiple targets.

The CPOs cell impenetrability substantiates the pharmacokinetic results obtained in our previous paper. The reported large apparent volume of distribution of **19** (JS-389) at steady-state Vss ~1650 mL.kg^{-1} may indicate that the drug is distributed into both the extracellular and intracellular fluids and tissues. Our visually confirmed finding regarding the impermeability of the CPOs into cells is in accord with their high polarity, because of which they are unlikely to permeate through biological membranes. Therefore, the alternative explanation put forward earlier, namely, the possibility of CPO's adsorption onto bone is gaining support. This assumption was tested on hydroxyapatite (HAP) as a model for bone.[26,50] There was found 44% absorption of **19** (JS-389) in 17 h, and 59% of the absorbed drug was resorbed in 15 h. This affinity of CPOs to bones is in accordance with a large Vss, as found in this case, making this compartment a depot providing reversible residence for the CPOs as shown by the long biological half-life (9 h, following intravenous administration). The extended half-life means that such a drug, in the clinical setting, which can be taken once daily, is the most convenient drug administration regimen for chronic therapy.

EXPERIMENTAL SECTION

Purity of Tested Compounds. All carbamoylphosphonates tested for carbonic anhydrase inhibition and reported in this article had been synthesized and purified to at least 95% as confirmed by combustion

analyses. These carbamoylphosphonates appear in the papers cited in Tables 1, 2, 3, and 4.

Chemical Syntheses. The fluorescent probes' syntheses are described in the Supporting Information.

Carbonic Anhydrase Inhibition. Assaying the CA-catalyzed CO_2 hydration activity was done by using an Applied Photophysics stopped-flow instrument as described previously.[51]

Permeability Experiments. Compounds A, B, and C have been dissolved in 1 mL of water in the presence of 15 mg sodium bicarbonate and tested at 2 mM final concentration.

HT-1080 and MDA-231 cells were exposed to the three compounds for 20 and 120 min at 37 °C. Following the incubation period, the cells were washed three times with PBS and visualized by a fluorescent inverted microscope. The results can be found in the Supporting Information.

Hypoxia. To induce the oxygen deprivation insult, HT-1080 and HeLa cells were introduced into a hypoxic device.[52] Briefly, the hypoxic device is composed of two connected chambers, maintained at 37 °C by circulating hot water into which Petri-dishes with HT-1080 and HeLa cells are placed. The air in the chambers is replaced with a mixture of 99% N_2 and 1% O_2 bubbled from a tank through a cylinder containing water, and passed through the system at a reduced flow rate to prevent evaporation of the medium. The oxygen level within the device is 1% (as measured online by an oxygen analyzer), representing a hypoxic insult. Control cells were kept in the humidified chamber in an atmosphere containing 21% O_2 (normoxia). The cells were treated with various concentrations of CPOs for 24 h (the whole oxygen deprivation period).

Cell death/damage was measured at the end of the hypoxia period by quantifying the activity of released LDH into the medium, using a commercial kit (Pointe, Canton, MI). LDH activity was determined spectrophotometrically at 340 nm by following the rate of conversion of oxidized nicotinamide adenine dinucleotide (NAD+) to the reduced form (NADH). The results can be found in Table 5.

Control cells, kept in normoxic conditions were not affected by the addition of CPOs to the culture media (up to 100 uM). LDH activity in the culture media increased in both cell lines in a dose dependent manner. (2.4-fold and 1.3-fold respectively).

■ ASSOCIATED CONTENT

Supporting Information

Table of kinetic parameters, permeability results, structures of fluorescent probes, cancer cells in the presence of fluorescent probes at 20 min and 2 h, as well as syntheses of the fluorescent probes used. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail addresses: elib@ekmd.huji.ac.il, eli.breuer@huji.ac.il.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported in part by the Grass Center for Drug Design and Synthesis of Novel Therapeutics to E.B., A. H., and R.R. A.H., R.R., and E.B. are affiliated with the David R. Bloom Center of Pharmacy and the Brettler Center for Pharmacology, in the School of Pharmacy, Faculty of Medicine, Hebrew University of Jerusalem. The authors thank Professor Philip Lazarovici and Ms. Adi Lahiani for their help with the hypoxia studies.

■ REFERENCES

(1) Carbamoylphosphonates, Part 10.

(2) (a) Li, N. G.; Shi, Z.-H.; Tang, Y. P.; Duan, J. A. Selective matrix metalloproteinase inhibitors for cancer. *Curr. Med. Chem.* **2009**, *16*, 3805−3827. (b) Bourguet, E.; Sapi, J.; Emonard, H.; Hornebeck, W. Control of melanoma invasiveness by anticollagenolytic agents: A reappraisal of an old concept. *Anti-Cancer Ag. Med. Chem.* **2009**, *9*, 576−597. (c) Friedl, P.; Wolf, K.; Travel, T. The role of proteases in individual and collective cancer cell invasion. *Cancer Res.* **2008**, *68*, 7247−7249. (d) Hu, J.; Van den Steen, P. E.; Sang, Q.-X.; Opdenakker, G. Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat. Rev. Drug Discovery* **2007**, *6*, 480−498.

(3) (a) Ahrens, D.; Koch, A. E.; Pope, R. M.; Steinpicarella, M.; Niedbala, M. J. Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. *Arthritis Rheumatism* **1996**, *39*, 1576. (b) Blaser, J.; Triebel, S.; Maasjosthusmann, U.; Romisch, J.; Krahlmateblowski, U.; Freudenberg, W.; Fricke, R.; Tschesche, H. Determination of metalloproteinases, plasminogen-activators and their inhibitors in the synovial fluids of patients with rheumatoid arthritis during chemical synoviorthesis. *Clin. Chim. Acta* **1996**, *244*, 17−33. (c) Bottomley, K. M.; Johnson, W. H.; Walter, D. S. Matrix metalloproteinase inhibitors in arthritis. *J. Enzyme Inhibit.* **1998**, *13*, 79−101.

(4) (a) Tezuka, K.; Nemato, K.; Tezuka, Y.; Sato, T.; Ikeda, Y.; Kobori, M.; Kawashima, H.; Eguchi, H.; Hakeda, Y.; Kumegawa, M. Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts. *J. Biol. Chem.* **1994**, *269*, 1106. (b) Ohishi, K.; Fujita, N.; Morinaga, Y.; Tsuruo, T. H-31 human breast cancer cells stimulate type I collagenase production in osteoblast-like cells and induce bone resorption. *Clin. Exp. Metastasis* **1995**, *13*, 287−295. (c) Witty, J. P.; Foster, S. A.; Stricklin, G. P.; Matrisian, L. M.; Stern, P. H. Parathyroid hormone-induced resorption in fetal rat limb bones is associated with production of the metalloproteinases collagenase and gelatinase B. *J. Bone Mineral Res.* **1996**, *11*, 72−78.

(5) Woessner, J. F., Jr. The family of matrix metalloproteinases. *Ann. N. Y. Acad. Sci.* **1994**, *732*, 11−21.

(6) Celentano, D. C.; Frishman, W. H. Matrix metalloproteinases and coronary artery disease: a novel therapeutic target. *J. Clin. Pharmacol.* **1997**, *37*, 991−1000.

(7) Lorenzl, P. R.; Koedel, S.; Sporer, U.; Vogel, B.; Frosch, U.; Pfister, M. Matrix metalloproteinases contribute to the blood-brain barrier disruption during bacterial meningitis. *Ann. Neurol.* **1998**, *44*, 592−600.

(8) (a) White, A. D.; Bocan, T. M.A.; Boxer, P, A.; Peterson, J. T.; Schrier, D. , Emerging therapeutic advances for the generation matrix metalloproteinase inhibitors. *Curr. Pharm. Design* **1997**, *3*, 45−58. (b) Armstrong, P. W.; Moe, G. W.; Howard, R. J.; Grima, E. A.; Cruz, T. F. Structural remodelling in heart failure: gelatinase induction. *Can. J. Cardiol.* **1994**, *10*, 214.

(9) Suzuki, R.; Miyazaki, Y.; Takagi, K.; Torii, K.; Taniguchi, H. Matrix metalloproteinases in the pathogenesis of asthma and COPD: implications for therapy. *Treat. Respir. Med.* **2004**, *3*, 17−27.

(10) Fukuda, E. M.; Nakano, Y.; Katano, I.; Fujimoto, Y.; Hayakawa, N. Serum levels of TIMP-2 and MMP-2 in patients with liver disease. *Liver* **1997**, *17*, 293−299.

(11) Romanic, A. M.; White, R. F.; Arleth, A. J.; Ohlstein, E. H.; Barone, F. C. Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size. *Stroke* **1998**, *29*, 1020−1030.

(12) Konstantinopoulos, P. A.; Karamouzis, M. V.; Papatsoris, A. G.; Papavassiliou, A. G. Matrix metalloproteinase inhibitors as anticancer agents. *Int. J. Biochem. Cell Biol.* **2008**, *40*, 1156−1168.

(13) 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. Topics Med. Chem* **2006**, *6*, 289−316.

(14) Krüger, A.; Kates, R. E.; Edwards, D. R. Avoiding spam in the proteolytic internet: Future strategies for anti-metastatic MMP inhibition. *Biochim. Biophys. Acta* **2010**, *1803*, 95−102.

(15) Engel, C. K.; Pirard, B.; Schimanski, S.; Kirsch, R.; Habermann, J.; Klingler, O.; Schlotte, V.; Weithmann, K. U.; Wendt, K. U. Structural basis for the highly selective inhibition of MMP-13. *Chem. Biol.* **2005**, *12*, 181−189.

(16) Pochetti, G.; Montanari, R.; Gege, C.; Chevrier, C.; Taveras, A. G.; Mazza, F. Extra binding region induced by non-zinc chelating inhibitors into the S1′ subsite of matrix metalloproteinase 8 (MMP-8). *J. Med. Chem.* **2009**, *52*, 1040−1049.
(17) Devel, L.; Rogakos, V.; David, A.; Makaritis, A.; Beau, F.; Cuniasse, P.; Yiotakis, A.; Dive, V. Development of selective inhibitors and substrate of matrix metalloproteinase-12. *J. Biol. Chem.* **2006**, *281*, 11152−11160.
(18) Veerendhar, A.; Reich, R.; Breuer, E. Phosphorus based inhibitors of matrix metalloproteinases. *C. R. Chimie* **2010**, *13*, 1191−1202.
(19) López-Otín, C.; Matrisian, L. M. Emerging roles of proteases in tumour suppression. *Nat. Rev. Cancer* **2007**, *7*, 800−808.
(20) Morrison, C. J.; Butler, G. S.; Rodriguez, D.; Overall, C. M. Matrix metalloproteinase proteomics: substrates, targets, and therapy. *Curr. Opin. Cell Biol.* **2009**, *21*, 645−653.
(21) Farkas, E.; Katz, Y.; Bhusare, S.; Reich, R.; Röschenthaler, G.-V.; 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.
(22) 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.
(23) 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.
(24) 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.
(25) Hoffman, A.; Qadri, B.; Frant, J.; Katz, Y.; Bhusare, S.; Breuer, E.; Hadar, R.; Reich, R. Carbamoylphosphonate Matrix Metalloproteinase Inhibitors 6. *cis*-2-Aminocyclohexylcarbamoyl phosphonic Acid. A Novel Orally Active Antimetastatic Matrix Metalloproteinase-2 Selective Inhibitor −Synthesis, and Pharmacodynamic and Pharmacokinetic Analysis. *J. Med. Chem.* **2008**, *51*, 1406−1414.
(26) Frant, J.; Veerendhar, A.; Chernilovsky, T.; Nedvetzki, S.; Vaksman, O.; Hoffman, A.; Breuer, E.; Reich, R. Carbamoylphosphonates 9 - Orally active, antimetastatic, non-toxic, diphenyl ether-derived carbamoylphosphonate matrix metalloproteinase inhibitors. *ChemMedChem* **2011**, *6*, 1471−1477.
(27) Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat. Rev. Drug Discovery* **2008**, *7*, 168−181.
(28) (a) Supuran, C. T. Carbonic anhydrases as drug targets − general presentation. In *Drug Design of Zinc-Enzyme Inhibitors: Functional, Structural, and Disease Applications*, Supuran, C. T., Winum, J.-Y., Eds.; Wiley: Hoboken, 2009; pp 15−38. (b) Winum, J.-Y.; Montero, J.-L.; Scozzafava, A.; Supuran, C. T. Zinc binding functions in the design of carbonic anhydrase inhibitors. In *Drug Design of Zinc-Enzyme Inhibitors: Functional, Structural, and Disease Applications*, Supuran, C. T., Winum, J.-Y., Eds.; Wiley: Hoboken, 2009; pp 39−72. (c) Alterio, V.; Di Fiore, A.; D'Ambrosio, K.; Supuran, C. T.; De Simone, G. X-Ray crystallography of CA inhibitors and its importance in drug design. In *Drug Design of Zinc-Enzyme Inhibitors: Functional, Structural, and Disease Applications*, Supuran, C. T., Winum, J.-Y., Eds.; Wiley: Hoboken, 2009; pp 73−138. (d) Alterio, V.; Di Fiore, A.; D'Ambrosio, K.; Supuran, C. T.; De Simone, G. Multiple Binding Modes of Inhibitors to Carbonic Anhydrases: How to Design Specific Drugs Targeting 15 Different Isoforms? *Chem. Rev.* **2012**, *112*, 4421−4468.
(29) Svastová, E.; Hulíková, A.; Rafajová, M.; Zat'ovicová, M.; Gibadulinová, A.; Casini, A.; Cecchi, A.; Scozzafava, A.; Supuran, C. T.; Pastorek, J.; Pastoreková, S. Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. *FEBS Lett.* **2004**, *577*, 439−445.
(30) Neri, D.; Supuran, C. T. Interfering with pH regulation in tumours as a therapeutic strategy. *Nat. Rev. Drug Discovery* **2011**, *10*, 767−777.
(31) McDonald, P. C.; Winum, J.-Y.; Supuran, C. T.; Dedhar, S. Recent Developments in Targeting Carbonic Anhydrase IX for Cancer Therapeutics. *Oncotarget* **2012**, *3*, 84−97.
(32) Lou, Y.; McDonald, P. C.; Oloumi, A.; Chia, S. K.; Ostlund, C.; Ahmadi, A.; Kyle, A.; Auf dem Keller, U.; Leung, S.; Huntsman, D. G.; Clarke, B.; Sutherland, B. W.; Waterhouse, D.; Bally, M. B.; Roskelley, C. D.; Overall, C. M.; Minchinton, A.; Pacchiano, F.; Carta, F.; Scozzafava, A.; Touisni, N.; Winum, J. Y.; Supuran, C. T.; Dedhar, S. Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Res.* **2011**, *71*, 3364−76.
(33) McIntyre, A.; Patiar, S.; Wigfield, S.; Li, J. L.; Ledaki, I.; Turley, H.; Leek, R.; Snell, C.; Gatter, K.; Sly, W. S.; Vaughan-Jones, R. D.; Swietach, P.; Harris, A. L. Carbonic anhydrase IX promotes tumor growth and necrosis in vivo and inhibition enhances anti-VEGF therapy. *Clin. Cancer Res.* **2012**, *18*, 3100−11.
(34) Chen, R.; Schlossman, A.; Breuer, E.; Hägele, G.; Tillmann, C.; Van Gelder, J. M.; Golomb, G. Long-Chain Functional Bisphosphonates: Synthesis, Anticalcification, and Antiresorption Activity. *Heteroatom Chem.* **2000**, *11*, 470−479.
(35) Harel, E.; Rubinstein, A.; Chen, W.; Breuer, E.; Tirosh, B. Aminoalkylcarbamoylphosphonates reduce TNFa release from activated immune cells. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6518−6523.
(36) Frant, J. Ph.D. Thesis; Hebrew University of Jerusalem, 2010.
(37) Cecchi, A.; Hulikova, A.; Pastorek, J.; Pastoreková, S.; Scozzafava, A.; Winum, J. Y.; Montero, J. L.; Supuran, C. T. Carbonic anhydrase inhibitors. Design of fluorescent sulfonamides as probes of tumor-associated carbonic anhydrase IX that inhibit isozyme IX-mediated acidification of hypoxic tumors. *J. Med. Chem.* **2005**, *48*, 4834−4841.
(38) Pastorekova, S.; Pastorek, J. in *Carbonic Anhydrase - Its Inhibitors and Activators,* Supuran, C. T., Ed.; CRC Press: Boca Raton, 2004; pp 255−281.
(39) Reich, R.; Blumenthal, M.; Liscovitch, M. Role of phospholipase D in laminin-induced production of gelatinase A (MMP-2) in metastatic cells. *Clin. Exp. Metastasis* **1995**, *13*, 134−140.
(40) Grant, D. S.; Tashiro, K.; Segui-Real, B.; Yamada, Y.; Martin, G. R.; Kleinman, H. K. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. *Cell* **1989**, *58*, 933−943.
(41) Stockwin, L. H.; Blonder, J.; Bumke, M. A.; Lucas, D. A.; Chan, K. C.; Conrads, T. P.; Issaq, H. J.; Veenstra, T. D.; Newton, D. L.; Rybak, S. M. Proteomic Analysis of Plasma Membrane from Hypoxia-Adapted Malignant Melanoma. *J. Proteome Res.* **2006**, *5*, 2996−3007.
(42) (a) Rusconi, S.; Innocenti, A.; Vullo, D.; Mastrolorenzo, A.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Interaction of isozymes I, II, IV, V, and IX with phosphates, carbamoyl phosphate, and the phosphonate antiviral drug foscarnet. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5763−5767. (b) Winum, J.-Y.; Innocenti, A.; Gagnard, V.; Montero, J.-L.; Scozzafava, A.; Vullo, D.; Supuran, C. T. Carbonic anhydrase inhibitors. Interaction of isozymes I, II, IV, V, and IX with organic phosphates and phosphonates. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1683−1686. (c) Temperini, C.; Innocenti, A.; Guerri, A.; Scozzafava, A.; Rusconi, S.; Supuran, C. T. Phosph(on)ate as a zinc-binding group in metalloenzyme inhibitors: X-ray crystal structure of the antiviral drug foscarnet complexed to human carbonic anhydrase. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2210−2215. (d) De Simone, G.; Supuran, C. T. (In)organic anions as carbonic anhydrase inhibitors. *J. Inorg. Biochem.* **2012**, *111*, 117−129.
(43) (a) Scozzafava, A.; Supuran, C. T. Carbonic Anhydrase and Matrix Metalloproteinase Inhibitors: Sulfonylated Amino Acid Hydroxamates with MMP Inhibitory Properties Act as Efficient Inhibitors of CA Isozymes I, II, and IV, and *N*-Hydroxysulfonamides Inhibit Both These Zinc Enzymes. *J. Med. Chem.* **2000**, *43*, 3677−3687. (b) Esteves, M. A.; Ortet, O.; Capelo, A.; Supuran, C. T.; Marques, S. M.; Santos, M. A. New hydroxypyrimidinone-containing sulfonamides as carbonic anhydrase inhibitors also acting as MMP inhibitors. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3623−3627.

(44) Renkiewicz, R.; Qiu, L.; Lesch, C.; Sun, X.; Devalaraja, R.; Cody, T.; Kaldjian, E.; Welgus, H.; Baragi, V. Broad-spectrum matrix metalloproteinase inhibitor marimastat-induced musculoskeletal side effects in rats. *Arthritis Rheum.* **2003**, *48*, 1742−1749.
(45) O'Brien, E. C.; Farkas, E.; Gil, M. J.; Fitzgerald, D.; Castineras, A.; Nolan, K. B. Metal complexes of salicylhydroxamic acid (H(2)Sha), anthranilic hydroxamic acid and benzohydroxamic acid. Crystal and molecular structure of [Cu(phen)(2)(Cl)] Cl center dot H(2)Sha, a model for a peroxidase-inhibitor complex. *J. Inorg. Biochem.* **2000**, *79*, 47−51.
(46) Breuer, E.; Frant, J.; Reich, R. Recent Non Hydroxamate Matrix Metalloproteinase Inhibitors. *Expert Opin. Ther. Patents* **2005**, *15*, 253−269.
(47) Goodsell, D. S. Biomolecules and Nanotechnology. *American Scientist* **2000**, *88*, 230.
(48) Ohlson, S. Designing transient binding drugs: A new concept for drug discovery. *Drug Discovery Today* **2008**, *13*, 433−439.
(49) It has been pointed out by the authors of the following paper 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.
(50) Jahnke, W.; Henry, C. An in vitro assay to measure targeted drug delivery to bone mineral. *ChemMedChem* **2010**, *5*, 770−776.
(51) Maresca, A.; Temperini, C.; Vu, H.; Pham, N. B.; Poulsen, S.-A..; Scozzafava, A.; Quinn, R. J.; Supuran, C. T. Non-Zinc Mediated Inhibition of Carbonic Anhydrases: Coumarins Are a New Class of Suicide Inhibitors. *J. Am. Chem. Soc.* **2009**, *131*, 3057−3062.
(52) Tabakman, R.; Lazarovici, P.; Kohen, R. Neuroprotective effects of carnosine and homocarnosine on pheochromocytoma PC12 cells exposed to ischemia. *J. Neurosci. Res.* **2002**, *68*, 463−469.