Journal of Enzyme Inhibition and Medicinal Chemistry

http://informahealthcare.com/enz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, Early Online: 1–6
 DOI: 10.3109/14756366.2014.968146

informa
healthcare

RESEARCH ARTICLE

Carbamoylphosphonates inhibit autotaxin and metastasis formation *in vivo*

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Abstract

Autotaxin is an extracellular, two zinc-centered enzyme that hydrolyzes lysophosphatidyl choline to lysophosphatidic acid, involved in various cancerous processes, e.g. migration, proliferation and tumor progression. We examined the autotaxin inhibitory properties of extended structure carbamoylphosphonates (CPOs) $PhOC_6H_4SO_2NH(CH_2)_nNHCOPO_3H_2$, with increasing lengths of methylene chains, $(CH_2)_n$, $n = 4$–8. Carbamoylphosphonates having $n = 6$, 7, 8 inhibited autotaxin *in vitro* with $IC_{50} \approx 1.5\,\mu M$. Using an imaging probe we demonstrated that compound $n = 6$ inhibits recombinant autotaxin activity *in vitro* and *in vivo*, following oral CPO administration. Additionally, daily oral administration of compound $n = 7$ inhibited over 90% of lung metastases in a murine melanoma metastasis model. Both the carbamoylphosphonates and the enzymes reside and interact in the extracellular space expecting minimal toxic side effects, and presenting a novel approach for inhibiting tumor proliferation and metastasis dissemination.

Keywords

Autotaxin, carbamoylphosphonate, carbonic anhydrase, matrix metalloproteinase

History

Received 25 August 2014
Accepted 18 September 2014
Published online 11 February 2015

Introduction

For cancer to thrive, it must first develop a suitable supporting environment such as the tumor microenvironment (TME) which enables tumor cells to express the enzymes that can support tumor proliferation or metastasis dissemination. In this current research we focused on three families of enzymes, which share in common having a zinc-atom and their ability to aid the progression of cancerous lesions and metastasis.

Studying the tumor microenvironment's extracellular zinc enzymes, autotaxin (ATX or ENPP2) is the core of this present research. This enzyme was first isolated as an autocrine motility factor from melanoma cells in 1992[1]. ATX is a secreted hydrolase, the activity of which originates from a threonine residue and from two zinc ions in the active site[2]. ATX belongs to the ectonucleotide pyrophosphatase and phosphodiesterase (ENPP) family, which consists of seven members[3]. ATX is the only member having lysophospholipase D (lysoPLD) activity, which hydrolyzes lysophosphatidyl choline (LPC) to the bioactive lysophosphatidic acid (LPA)[4]. The latter is involved in diverse malignant processes such as migration, proliferation and differentiation[5]. ATX is at the focus of considerable effort to achieve clinically useful inhibitors, however so far without success[6]. Our research focuses on the inhibiting properties of carbamoylphosphonates (CPO) towards ATX.

In this paper, we show that *N-6-*[*4*-phenoxybenzenesulfonamido]hexyl-carbamoylphosphonate (CPO $n = 6$) inhibits recombinant autotaxin (ATX) *in vitro*. Furthermore, orally administered CPO $n = 6$ inhibited ATX *in vivo*, as established by ATX-Red-2 in an *in vivo* imaging probe visualizing enzymatic activity in SCID mice, bearing a triple-negative cell lineMDA231-induced breast tumor.

In addition to the CPOs' activity on ATX, we investigated the enzymatic inhibiting properties of CPO $n = 7$, 8 on carbonic anhydrase (CA) IX and XII. These enzymes belong to the family of zinc metalloenzymes which facilitate the formation of bicarbonate from carbon dioxide and water[7]. The relevance of CAs to cancer was discovered in 1992, with the identification of CAIX and CAXII[8]. These isoforms are barely expressed in normal tissues, but are predominantly associated with and overexpressed in tumors and are involved in critical processes related to cancer progression[9–11]. CAIX and XII are membrane-type isoforms with extracellularly exposed active sites, induced by the transcription factor Hypoxia Induced Factor 1α (HIF-1α). Their upregulation results in a decrease of extracellular pH, and is strongly associated with cancer cell survival and malignant progression[12,13]. The activity of CAIX is also important for metastasis, as recently demonstrated by Lou et al.[14], who showed that CAIX is an essential factor in the survival of tumor cells in hypoxic areas of breast tumor, and its activity contributes to metastasis generation in breast cancer. Furthermore, both inhibition of CAIX activity

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and depletion of CAIX expression have been shown to result in regression or growth inhibition of mouse and human breast tumors, as well as inhibition of cancer metastasis[15].

In the current research we report the inhibiting effects of CPOs $n = 7$, 8 on CA, primarily the cancer supporting isoforms CAIX and CAXII. This is an extension of recently reported research, which showed that CPOs $n = 5$, 6 – previously recognized *in vivo* as active anti-metastatic matrix metallo-proteinase (MMP) inhibitors[16–18] – also act as CA inhibitors[15].

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. MMPs expression is increased in tumors due to transcriptional changes: the secreted MMP 2 and 9 are synthesized by stromal cells, and their expression is upregulated by cancer cells[19]. In spite of extensive research, no clinically useful inhibitor has yet been identified for MMPs. Their efficient inhibition is, therefore, an important therapeutic target[20].

Experimental section

Chemical synthesis

CPOs used in this work have been synthesized previously[18].

Inhibitor kinetics

MMPs' and CAs' inhibitory constants have been determined as previously described, respectively[15,18].

In vitro studies of ATX activity

ATX activity was assessed according to manufacturer instructions, by a colorimetric assay (Cayman Chemical, Ann Arbor MI, Kit # 700580), where recombinant ATX cleaves *bis*-(*p*-nitrophenyl) phosphate to liberate *p*-nitrophenol measured at 405–415 nm, in the presence of various putative inhibitors.

In vivo studies

ATX activity in vivo

ATX activity *in vivo* was estimated using the ATX-Red-2 *in vivo* imaging probe for the visualization of enzymatic activity (Echelon Bioscience, Salt Lake City, UT, CAT # L-2010)[21]. ATX-Red-2 is an analog of LPC and contains a near-infrared fluor and a quencher. The compound was injected to SCID mice bearing an MDA-231 cell-induced breast tumor, at a final dose of 0.5 mg/kg. CPO $n = 6$, an ATX inhibitor was administered *per os* at a single dose of 50 mg/kg. Fluorescence imaging was monitored at 6, 24 and 48 h following probe and drug administration by an IVIS *in vivo* imaging system.

Murine melanoma model

C57Bl mice aged 4–6 weeks weighing 20–25 g were used in the melanoma experiments. Experimental metastasis was studied in the murine melanoma model. In this model, B16F10 tumor cells (~50 000) were injected into the tail vein of C57Bl female mice. Eight mice were used for each CPO examined. The inhibitors were administered either PO or IP at 12.5 and 25 mg/kg doses. Mice were monitored for toxic symptoms. After 21 days, the animals were sacrificed, and the metastases formed on their lungs were counted after appropriate fixation.

Animal care

The joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center in Jerusalem, Israel, approved the study protocol for animal welfare (number MD-09-11910-5). The Hebrew University is an AAALAC International accredited institute.

Absorption and resorption studies of CPO on hydroxyapatite

A solution of the carbamoylphosphonic acid (CPO) (50 μM; in 100 mM Tris buffer; pH 7.5) was used. Three aliquots (3×4 mL) from the resulting solution were incubated with HAP (1.6 mg) at 37 °C at 170 rpm for 1 h. The remaining CPO solution was used as reference as well as for 2 h and 18 h absorption studies. After 1 h, the three aliquots were centrifuged (10 min, 2600*g*), and the UV absorbances of the clear solutions were recorded and subtracted from the initial value to obtain the amount of the absorbed CPOs. The same procedure was used to determine the absorbances after the time intervals of 2 h and 18 h, also in triplicates. The concentrations of CPO were calculated from the observed optical densities of the solutions.

To examine the reversibility of the absorption of CPOs to HAP, the samples from the 2 h absorption experiments were taken and centrifuged (5 min, 2600*g*). The HAP samples were isolated by decantation, then double-distilled water was added (2×2 ml) followed by centrifugation. The aqueous layer was decanted and the white precipitate was stirred with 4 mL Tris buffer (50 mM; pH 7.5,) at 37 °C at 170 rpm for 1 h, 2 h and 18 h. After centrifugation (10 min, 2600*g*), the supernatant solutions were removed, and their UV absorbance was recorded. The data are listed in the bottom part of Table 3. The absorbance was measured at $\lambda = 242$ nm, where the absorbance is maximum for CPOs.

Results

Inhibitor kinetics

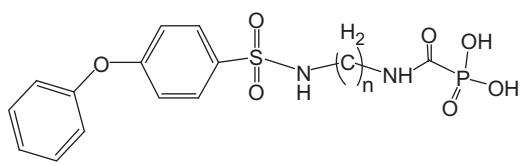
CA inhibiting effect of CPO $n = 5$–8 are shown in Table 1. All the compounds exhibit similar pharmacological inhibiting profiles, displaying $IC_{50} < 10$uM for both CA IX and XII.

MMP 2 inhibiting profiles of CPO $n = 5$–8 are also depicted in Table 1. While CPOs $n = 5$, 6 exhibit significant inhibiting effects of IC50 < 5uM, CPO $n = 7$, 8 did not show any inhibiting effects ($IC_{50} > 100$ μM). This is since the MMP's S1' pocket has limited depth and it is unable to accommodate the longer linking chain.

In vitro studies

The CPOs examined in this article have similar structures to some published *in vitro* active ATX inhibitors[6]. Table 1 also depicts the inhibitory constants of the CPOs toward ATX. While CPO $n = 5$

Table 1. Inhibition constants of CPOs on MMP 2, CA IX and XII and ATX enzymes.*†



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		CPO, *n*=			
		5	6	7	8
MMP	2	4	4	>100	>100
CA	IX	6.3	7	0.86	0.68
	XII	5.7	6.2	1.4	0.78
ATX		>100	1.5	1.5	1.5

*IC_{50} units are μM.
†MMP-9 $IC_{50} > 100$ for CPO $n = 5$–8.

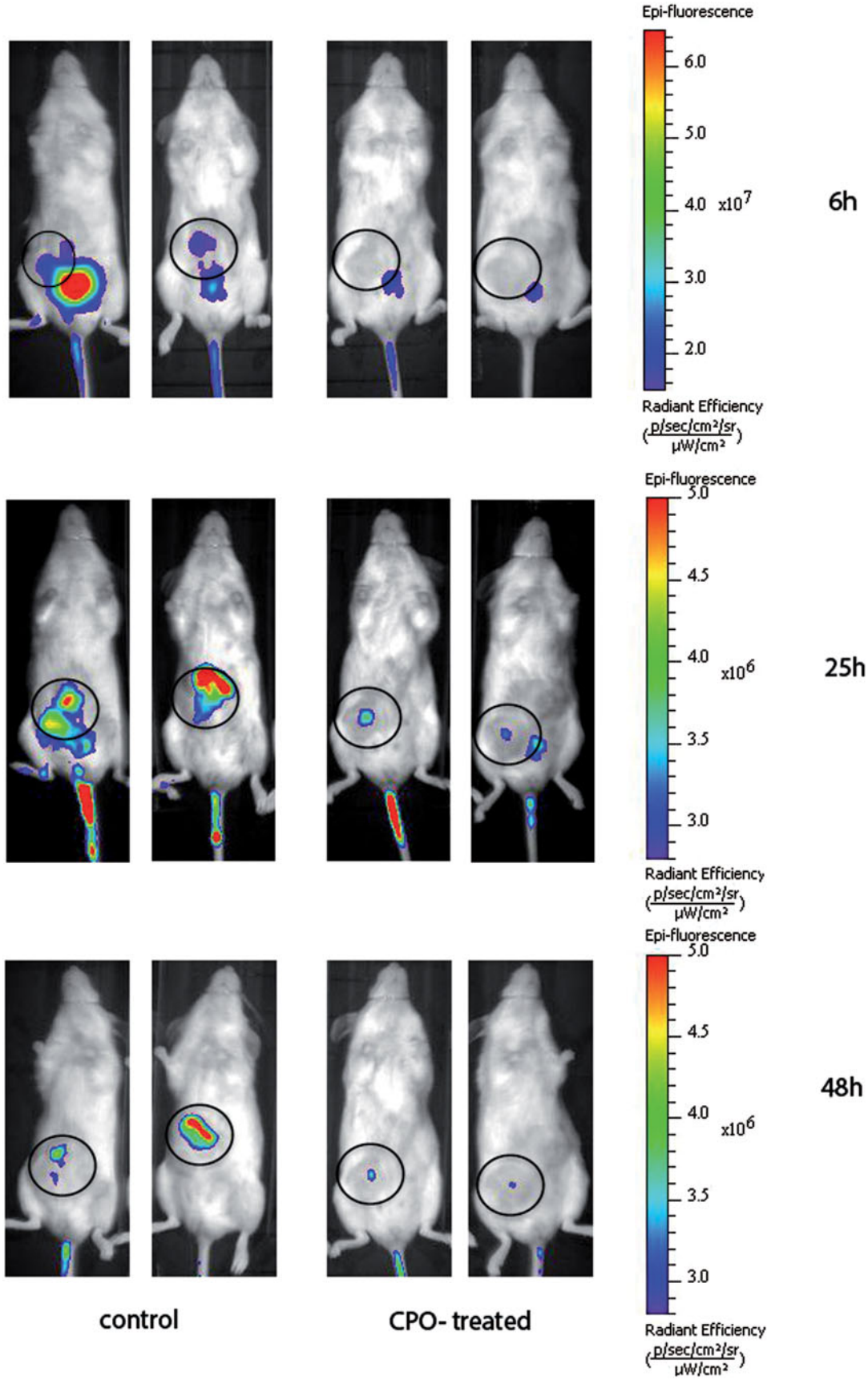


Figure 1. The fluorescent image of the experimental animals at the different time points. ATX activity in vivo was estimated by the using ATX-Red-2, an *in-vivo* imaging probe, at a final dose of 0.5 mg/kg, for the visualization of enzymatic activity. The data were collected at three time points as indicated. Circle shows the tumor location.

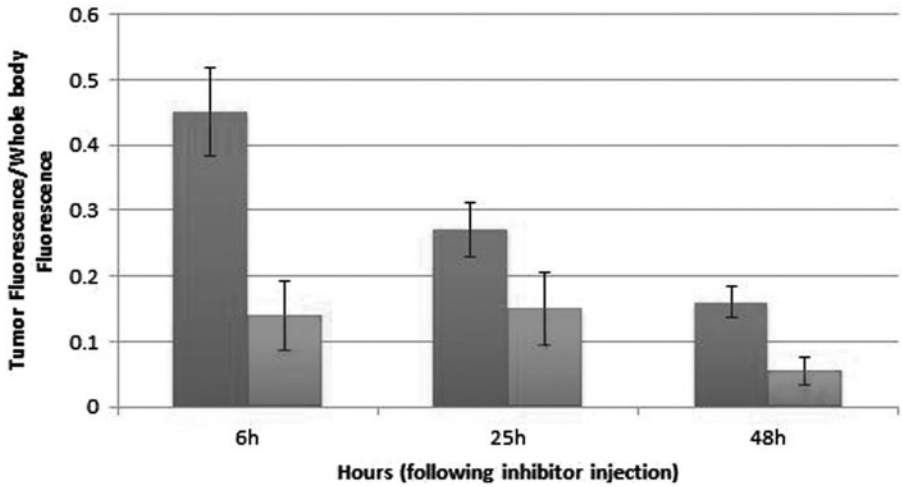


Figure 2. The fluorescence ratio of the tumor versus the whole body fluorescence, as a function of time. There is a gradual decrease in the amount of fluorescence due to dye clearance from the mice, as time progresses. Dark grey bars represent the control mice while the light grey bars represent the treated ones.

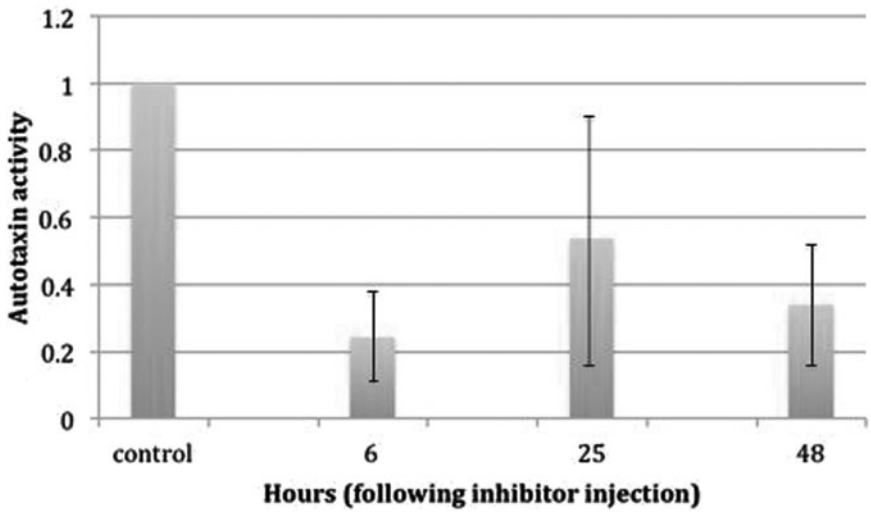


Figure 3. The ATX activity as function of time in the treated mice. It is important to point out that ATX activity is still inhibited over 60% at 48 h following the administration of a single dose of the drug.

is too short to inhibit ATX ($IC_{50} = 100$), the three longest structures of CPOs $n = 6$, 7, 8 were found to be active ATX inhibitors at low micromolar IC_{50}.

Despite the apparent similarity between the CPOs' chemical structures, they differ in ATX-inhibiting profiles. This demonstrates the importance of specific structural requirements and the selectivity it imposes.

In vivo studies

ATX activity in vivo

ATX activity *in vivo* was estimated using ATX-Red-2-*in-vivo* imaging probe for the visualization of enzymatic activity in SCID mice, bearing a triple-negative cell line MDA231-induced breast tumor (Figure 1). Focal intensity of the fluorescence of the probe indicates the *in vivo* activity of ATX enzyme at the site of the tumor. A single oral dose administration of CPO $n = 6$ resulted in diminished fluorescence intensity at the tumor sites, indicating inhibition of ATX by the compound. When not inhibited (i.e. control group), the enzymatic activity of ATX was measurable even at 48 h after probe injection. At the same time point, the inhibitory effect of CPO $n = 6$ was significantly evident (Figures 2 and 3).

Antimetastatic studies: murine melanoma model

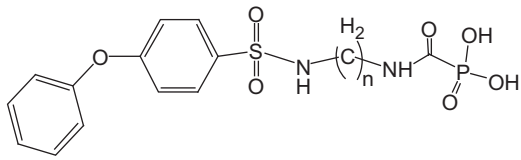
Mice were injected intravenously with B16F10 cells and treated daily for three weeks by CPOs $n = 6$, 7 as indicated in a murine melanoma model. CPO $n = 7$ was examined when given orally, and compared with the previously published effect of either oral or intraperitoneal administration of CPO $n = 6$ (Table 2).

It is evident that daily treatment of the examined CPOs is effective in reducing metastasis when administered orally or intraperitoneally. This correlates well with the enzyme-inhibiting properties displayed above and the long pharmacokinetic half-life previously reported.

Absorption and resorption studies of CPO on hydroxyapatite

The pharmacokinetic examination of CPOs $n = 5$, 6 reported in our previous publication[18] shows volumes of distribution which considerably exceeded the volumes of the extracellular fluid (EF) in rat, indicating that in addition to its presence in the EF, a significant proportion of the drug may be accumulated in certain organs. In this work we examined the extent of CPO $n = 6$, 7 absorption using a recently published method for studying bisphosphonate absorption to hydroxyapatite (HAP) as a model

Table 2. *In vivo* effect of selected CPO inhibitors on metastasis formation in mice.*



CPO Inhibitor symbol	Mode of introduction	CH2 chain length n	Dose 12.5 mg/kg % decrease in metastases relative to control	Dose 25 mg/kg % decrease in metastases relative to control
JS-403	IP	6	82	82
JS-403	PO	6	61	71
TCH-18	PO	7	67	90

*Mice were injected IV with B16F10 cells and treated daily for three weeks by the drugs; values reflect percent decrease in lung metastases relative to control.

Table 3. Carbamoylphosphonates absorption to (A), and resorption from (B) hydroxyapatite (HAP).

Time (h)	CPO $n=5$	CPO $n=6$	CPO $n=7$
Absorption (A) of CPOs to HAP (%)			
0	0	0	0
1	33	37	57
2	35	49	59
18	41	51	67
Resorption (B) of CPOs from HAP (%)*			
0	0	0	0
1	33	29	14
2	36	33	16
18	39	37	18
∞	100	100	100

*Samples for the resorption studies were taken from the 2 h absorption experiments.

for bone[22], and compared it with CPO $n=5$ which was re-examined. As shown in Table 3, the absorption of CPOs to HAP increases as the lengths of the aliphatic chains increase. In contrast, the rate of resorption of CPOs from HAP decreases in the homologous series from CPO $n=5$ to $n=7$, as observed for alkyl bisphosphonates in bone-resorption[23].

Discussion

In the course of their growth, tumors avoid the inhibitory signals from the adjacent cells and subjugate the surrounding cells to their own needs. This results in unlimited proliferation, invasion into neighboring tissues and ultimate establishment of distant metastases. One characteristic feature of such microenvironment is a more acidic extracellular pH than that of normal tissues[24]. This higher lactate production by tumors in the presence of oxygen provides a growth advantage for tumor cells *in vivo*. One of the tumor cells' responses to acidosis and hypoxia is the induction of certain genes. Of note is the HIF-1α gene, which in turn induces the expression of numerous additional genes, such as ATX, which hydrolyzes LPC to the bioactive lipid LPA[4].

High expression of ATX is found in various tumor cell types, including neuroblastoma, hepatocellular carcinoma, breast cancer, renal cell carcinoma, glioblastoma, non-small cell lung cancer, B cell lymphomas, and thyroid carcinoma. Therefore, the inhibition of this membrane-bound enzyme is essential for abolishing the growing tumor. To our best knowledge, no ATX inhibitor reached clinical trial so far.

Similar to ATX, CA IX and XII are also induced by HIF-1α[13]. In the present context we focus on the inhibition properties of CPOs on these two CAs, CA IX and XII, which are membrane-type isoforms with extracellularly exposed active sites. Isozymes CA IX and CA XII are transmembranal enzymes and are critical for cell proliferation; their up-regulation is also strongly associated with cancer cell survival and malignant progression[12].

Using various breast cancer cell lines[14], it has been recently demonstrated that CAIX is essential to the survival of tumor cells under hypoxic conditions of breast tumor, and its activity contributes to metastasis generation. It has been proposed that CAIX could serve as a specific biomarker for this kind of tumor. The necessity of CAIX for tumor and metastasis formation has also been recently demonstrated by using the knock-down approach[25]. 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[25].

MMPs aid tumor progression by increasing cancer cell growth, migration and invasion, angiogenesis and metastasis[26,27]. In recent years, we have reported in a series of papers that show CPOs are able to act both *in vitro* and *in vivo* as inhibitors of the extracellular MMP 2[16–18] and possess zinc-binding ability[28].

The three enzyme families (ATX, CA IX and XII and MMP 2) have significant differences in their substrates and their products, but share similarities in the fact that they all have zinc ions at their catalytic sites and furthermore, they function in the extracellular medium. In light of this, we trust that our proposed CPOs, which exhibit a dual or triple inhibiting effect on these enzymes, as shown in Table 1, display anticancer and antimetastatic activities.

The duration of the ATX inhibition for more than 48 h after a single administration of CPO $n=6$ coincides with the biological half-life of this compound, measured in rats to be over 14 h[18]. Such lengthy activity in rodents may indicate an even longer duration in humans due to the differences in metabolic rates.

As suggested earlier[18], the biological activity of the CPOs is the result of a certain threshold inhibitory concentration required for the inhibition of the extracellular zinc enzymes. This explains the evident activity seen following oral treatment, despite the relatively poor oral bioavailability (0.4%)[18]. It is also in accordance with the relatively similar activity seen for CPO $n=6$, 7 following PO and IP administrations. The prolonged activity of the CPOs seen at this preclinical stage indicates the suitability of a once a day treatment (either as anti-metastatic or antitumoral drugs) that also seem to exhibit flexibility in case of poor compliance[29].

Acknowledgements

We highly appreciate Yael Ben-Nun's assistance with imaging analysis.

Declaration of interest

The authors declare no competing financial interest. This work was supported in part by the Grass Center for Drug Design and Synthesis of Novel Therapeutics to E.B. 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, The Hebrew University of Jerusalem.

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