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Synthesis and pharmacological evaluation of the stereoisomers of 3-carba cyclic-phosphatidic acid

Renuka Gupte [a], Anjaih Siddam [a], Yan Lu [a], Wei Li [a], Yuko Fujiwara [b], Nattapon Panupinthu [d], Truc-Chi Pham [c], Daniel L. Baker [c], Abby L. Parrill [c], Mari Gotoh [e], Kimiko Murakami-Murofushi [e], Susumu Kobayashi [f], Gordon B. Mills [d], Gabor Tigyi [b], Duane D. Miller [a,*]

[a] Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN 38163, USA
[b] Department of Physiology, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA
[c] Department of Chemistry, University of Memphis, TN 38152, USA
[d] Department of Systems Biology, M. D. Anderson Cancer Center, The University of Texas, Houston, TX 77054, USA
[e] Department of Biology, Ochanomizu University, Tokyo 112-8610, Japan
[f] Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba 278-8510, Japan

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ABSTRACT

Cyclic phosphatidic acid (CPA) is a naturally occurring analog of lysophosphatidic acid (LPA) in which the sn-2 hydroxy group forms a five-membered ring with the sn-3 phosphate. Here, we describe the synthesis of **R-3-CCPA** and **S-3-CCPA** along with their pharmacological properties as inhibitors of lysophospholipase D/autotaxin, agonists of the LPA_5 GPCR, and blockers of lung metastasis of B16-F10 melanoma cells in a C57BL/6 mouse model. **S-3CCPA** was significantly more efficacious in the activation of LPA_5 compared to the *R*-stereoisomer. In contrast, no stereoselective differences were found between the two isomers toward the inhibition of autotaxin or lung metastasis of B16-F10 melanoma cells in vivo. These results extend the potential utility of these compounds as potential lead compounds warranting evaluation as cancer therapeutics.

Lysophosphatidic acid (LPA) is a pleiotrophic phospholipid growth factor with multiple roles in cancer metastasis and progression.[1] LPA elicits numerous biological effects including the promotion of cellular survival, mitogenesis, angiogenesis, migration, and cancer invasion that are mediated, at least in part, by specific cell surface G-protein coupled receptors (GPCR) and intracellular targets that include the nuclear hormone receptor peroxisome proliferator-activated receptor (PPARγ).[2] Cylic-phosphatidic acid (1-acyl-2,3-glycerophosphate, CPA) is a naturally occurring analog of LPA in which the *sn*-2 hydroxy group forms a five-membered ring with the *sn*-3 phosphate.[3] CPA affects numerous cellular functions, including inhibition of cell cycle progression, induction of stress fiber formation, inhibition of tumor cell invasion and metastasis, and regulation of differentiation and survival of neuronal cells.[4] CPA is a weak agonist of the LPA_1 and LPA_2 GPCR.[5] Substitution of the *sn*-2 or *sn*-3 oxygen with a methylene in CPA yields carba-CPA (CCPA), a stabilized analog of CPA.[6] Previous work has shown that 3-CCPA does not activate the LPA_{1-4} GPCR[5] but is a weak agonist of LPA_5.[7]

Autotaxin (ATX) was initially identified as an autocrine tumor cell motility factor from melanoma cell conditioned medium.[8] ATX has lysophospholipase D enzyme activity and is responsible for the hydrolysis of lysophophatidylcholine leading to the generation of LPA[9,10] and CPA.[11] While ATX can also produce sphingosine-1-phosphate (S1P) in vitro, it does not appear to contribute in a major way to S1P production in vivo. High levels of autotaxin are generated by a wide variety of metastatic human tumor cell lines including human teratocarcinoma,[12] hepatocellularcarcinoma,[13] metastatic breast cancer,[1] ovariancancer,[14] thyroid carcinoma,[15] prostate cancer,[16] follicular lymphoma[17] and glioblastomamultiforme.[18] ATX also plays an important role in the chemotherapeutic resistance of breast[19] and ovarian cancer cells[14] to chemotherapeutic agents. ATX is under feedback inhibition by its hydrolytic products LPA, CPA, and sphingosine-1-phosphate (S1P).[20] Racemic 2-CCPA and 3-CCPA are potent inhibitors of ATX activity and 3-CCPA has been shown to reduce lung metastasis of B16-F10 melanoma cells injected intravenously into C57BL/6 mice.[5] To further explore the therapeutic utility of 3-CCPA, stereochemically pure isomers are needed.

Abbreviations: ATX, autotaxin; BSA, bovine serum albumin; CCPA, carbacyclic phosphatidic acid; CPA, cyclic phosphatidic acid; DIC, di-isopropyl carbodiimide; DMAP, dimethyl amino pyridine; GPCR, G-protein coupled receptors; HRMS, high resolution mass spectrometry; LPA, lysophosphatidic acid; NMR, nuclear magnetic resonance; PPTS, pyridinium-*p*-toluene sulfonate; TMSBr, trimethyl silyl bromide.

* Corresponding author. Tel.: +1 901 448 6026; fax: +1 901 448 3446.
E-mail address: dmiller@uthsc.edu (D.D. Miller).

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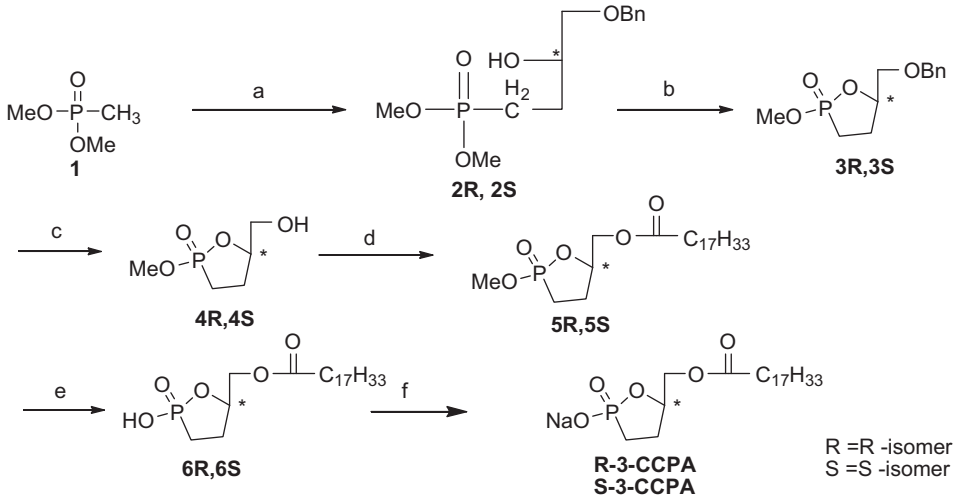

Scheme 1. Reagents and conditions: (a) (i) THF, *n*-BuLi (2.5 M in hexane), −78 °C, 0.5 h; (ii) *R*-benzyl glycidyl ether (*R*)/*S*-Benzyl glycidyl ether (*S*); (iii) THF, BF_3OET_2, −78 °C, 2 h, 68%; (b) PPTS, toluene, reflux, 5 h, 65%; (c) H_2, $Pd(OH)_2$/C,MeOH, 82.5%; (d) $C_{17}H_{33}COOH$, DMAP, DIC, DCM, 18 h, 78%; (e)TMSBr, CH_2Cl_2, 1 h 53%; (f) 0.005 M NaOH.

For this reason we describe the synthesis and characterization of both the **R-3-CCPA** and **S-3-CCPA**.

The approach used for the synthesis of the two stereoisomers of 3-CCPA is shown in Scheme 1. Dimethylphosphonate derivatives **2R** and **2S** were generated from compound **1** using *n*-butyllithium, BF_3·etherate and either the *R*- or *S*-isomer of benzylglycidyl ether. The corresponding 3-carbacyclic analogs **3R** and **3S** resulted from treatment with pyridinium *p*-toluenesulfonate (PPTS). Following benzyl group deprotection by hydrogenation, the resulting alcohols **4R** and **4S** were converted to oleoyl esters **5R** and **5S** using *N*,*N*′-diisopropylcarbodiimide (DIC) and dimethylaminopyridine (DMAP). Final products **R-3-CCPA** and **S-3-CCPA** were prepared by methyl group deprotection and conversion to the corresponding sodium salts using TMSBr and dilute NaOH, respectively. Optical rotations were determined by dissolving the compounds in methanol, to be +7.3° for **R-3-CCPA** and for **S-3-CCPA**, the optical rotation was −7.9°. Each compound was purified by silica gel column chromatography and verified by mass spectrometry, NMR and HRMS.

Compounds **R-3-CCPA** and **S-3-CCPA** were examined for their ability to block ATX-mediated hydrolysis of FS-3 (Echelon Biosciences, Inc., Salt Lake City, UT) using a fluorescence resonance energy transfer-based assay.[21] Recombinant ATX (25 nmol) in the presence of various concentrations of **R-3-CCPA**, **S-3-CCPA**, or LPA 18:1 (positive control) in assay buffer consisting of 1 mM $MgCl_2$, 1 mM $CaCl_2$, 3 mM KCl, 140 mM aCl, 50 mM Tris–HCl, pH 8.0 and 15 μM fatty acid free BSA was added to FS-3 (final concentration 1 μM). Assays were carried out in white wall 96-well plates (Corning Inc., Corning, NY) and the fluorescence (excitation 485 nm, emission 538 nm) was measured at the beginning and after 2 h of incubation at 37 °C using a FLEX station II plate reader (Molecular Devices, Sunnyvale, CA). Data were normalized to the corresponding vehicle control, and the mean ± standard deviation of triplicate wells was used to calculate ATX activity as per cent of vehicle control. The dose–response relationship of ATX inhibition showed little difference between the **R-3-CCPA**, **S-3-CCPA** or the racemate (Fig. 1). However, **R-3-CCPA** was approximately twofold more potent in this assay than **S-3-CCPA**. The kinetic mechanism by which **R-3-CCPA** and **S-3-CCPA** inhibited recombinant ATX-mediated hydrolysis of FS-3 were determined by varying the concentration of the substrate (FS-3, ranging from 0.3 to 20 μM) in the presence of three concentrations of each inhibitor (0, 0.5 and 2 times the IC_{50}). Simultaneous non-linear regression

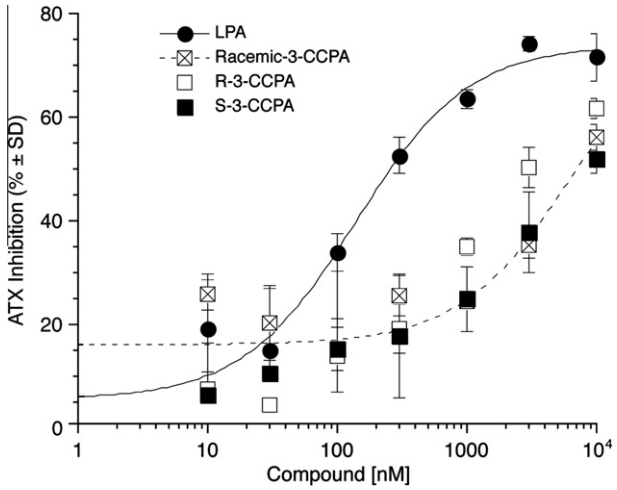

Figure 1. Dose–response relationship of ATX inhibition by LPA, **R-3-CCPA**, **S-3-CCPA** and racemic-3-CCPA analogs.

using WinNonLin® 6.1 (Pharsight, Mountain View, CA) was used to fit experimental data and calculate K_i and K_i' using the Michaelis–Menten equations for competitive, uncompetitive, mixed-mode, and non-competitive inhibition as we have described in recent work.[22–24] Mechanism of inhibition was assigned based on the lowest averaged percent residuals for each mechanism derived from curve fitting. Using this procedure **R-3-CCPA** and **S-3-CCPA** were determined to be mixed-mode ATX inhibitors with K_i values of 0.8 and 1.6 μM, respectively.

The lack of ligand stereospecificity of the LPA_1, LPA_2, and LPA_3 receptors has been published previously[25] but no information of stereoselective ligand activation for LPA_5 is currently available at the present time. **Racemic-3-CCPA** has previously been shown to bean agonist of the LPA_5 GPCR.[7] Here, we compared the dose–response curves of LPA_5 activation for **R-3-CCPA** and **S-3-CCPA** with that of the racemate. These experiments were performed in B103 cells stably expressing LPA_5. Wild type B103 cells do not produce Ca^{2+} transients in response to LPA and are widely used as a host cell for LPA receptor expression studies. B103-LPA_5 cells were loaded with Fura-2AM for 30 min in modified Krebs buffer containing 2% (v/v) pluronic acid, rinsed with Krebs buffer, and changes in the intracellular Ca^{2+} concentration were monitored by determining

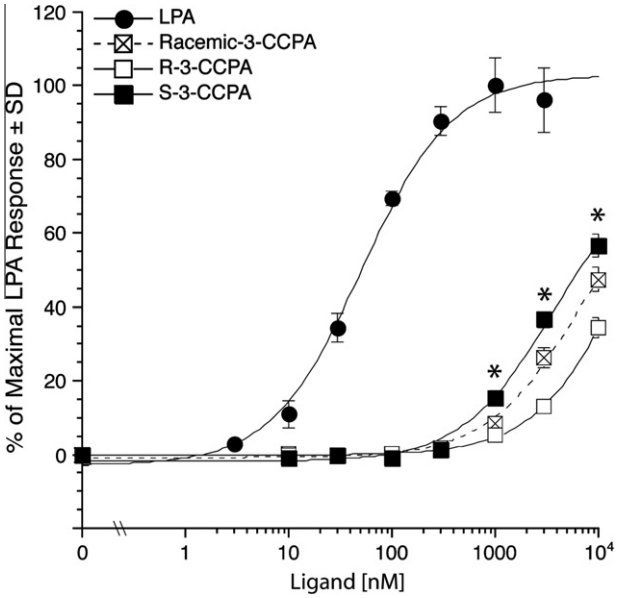

Figure 2. Dose–response relationship of LPA_5 mediated.

the ratio of emitted light intensities at 520 nm in response to excitation at 340 and 380 nm using a FLEX station II plate reader (Molecular Devices, Sunnyvale, CA).[26] Compound **S-3-CCPA** showed significantly higher (p <0.05) efficacy than did **R-3-CCPA** for LPA5-mediated calcium mobilization at concentrations above 1 μM (Fig. 2). Thus, the LPA_5 receptor shows a slight stereoselectivity for the *S*- over the *R*-stereoisomer which contrasts the weak preference (~2-fold) shown by ATX for the *R*-isomer.

We have previously shown that racemic 3-CCPA inhibited lung metastasis of B16-F10 melanoma in a mice model. To extend this observation, the stereoisomers were characterized in this model.[4,5] Eight-week-old female C57Bl/6 mice were inoculated with 5×10^4 melanoma cells via the tail vein and divided randomly into four groups. The groups then received either saline vehicle, **R-3-CCPA**, **S-3-CCPA**, or racemate (at 0.5 mg/kg intraperitoneally) 30 min after the B16-F10 inoculation and daily for an additional 10 days. Animals in all groups were monitored for an additional 10 days without further treatments. On day 21, all mice were sacrificed and lungs were dissected, fixed with formalin and the numbers of black melanoma nodules on the lung surface were counted in each sample (Fig. 3). All 3-CCPA treated groups (**R-3-CCPA**, **S-3-CCPA** and **Racemic-3-CCPA**) significantly reduced the number of lung metastases compared to the vehicle treated group. However, no statistically significant differences were found between the 3-CCPA treated groups using ANOVA followed by Newman–Keuls multiple comparison test.

In conclusion, we have synthesized pure stereoisomers of 3-CCPA and found that they inhibited ATX in vitro and B16-F10 melanoma metastasis in vivo without significant stereochemical preference. The lack of stereoselectivity is underlined by the equal

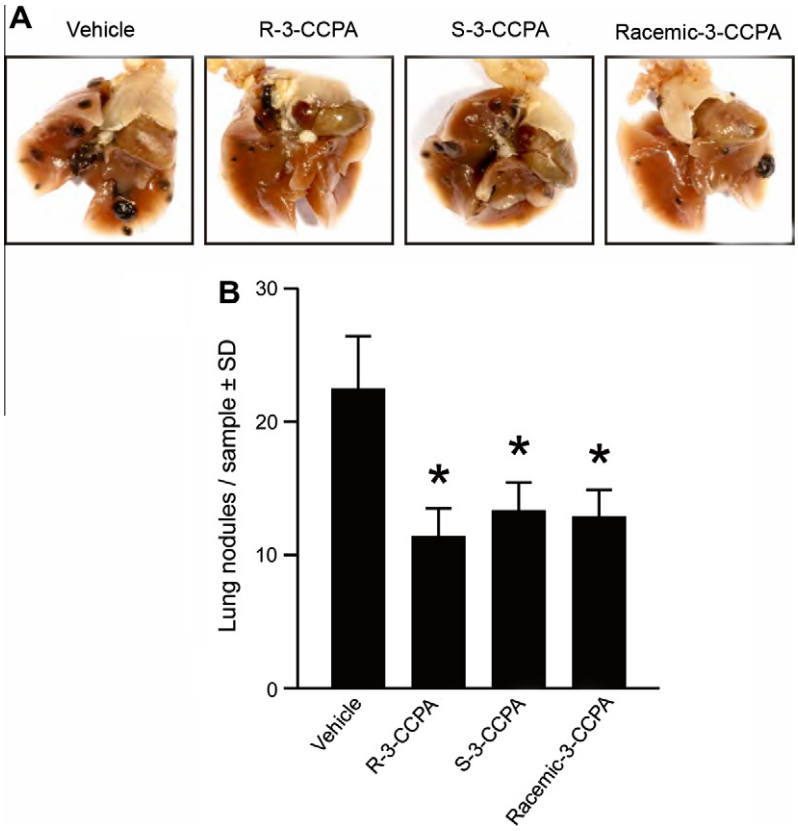

Figure 3. Lack of stereoselectivity in lung metastasis of B16-F10 melanoma cells by **R-3-CCPA**, **S-3-CCPA** and racemic-3-CCPA analogs in a mouse model. (A) Representative images show fixed intrathoracic organs including lung lobules with visible nodules on the surfaces in black. Total numbers of nodules were reduced in samples treated with 7*R*,7*S* or racemate compared to vehicle. Scale bar is 0.5 cm. (B) Lung nodules of B16-F10 melanoma cells were quantified. The number of lung nodules was significantly decreased in groups treated with **R-3-CCPA**, **S-3-CCPA**, and the Racemic-3-CCPA compared to vehicle. However, no statistically significant differences were found either between the stereoisomers or the racemate. Data represent the mean ± SEM, n = 6–8 mice. * p <0.05 compared to vehicle analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test.

efficacy of the racemic mixture. Interestingly, at the LPA_5 GPCR the *S*-stereoisomer (**S-3-CCPA**) showed significantly higher efficacy. This is the first indication that the LPA_5 receptor, unlike the $LPA_{1,2,3}$ receptors shows stereoselective activation by CCPA ligands.

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