Published in final edited form as:
Bioorg Med Chem Lett. 2010 December 15; 20(24): 7525–7528. doi:10.1016/j.bmcl.2010.09.115.

Synthesis and Pharmacological Evaluation of the Stereoisomersof 3-Carba Cyclic-Phosphatidic Acid

Renuka Gupte[a], **Anjaih Siddam**[a], **Yan Lu**[a], **Wei Li**[a], **Yuko Fujiwara**[b], **Nattapon Panupinthu**[d], **Truc-ChiPham**[c], **Daniel L. Baker**[c], **Abby L. Parrill**[c], **Mari Gotoh**, **Kimiko Murakami-Murofushi**[e], **Gordon B. Mills**[d], **Gabor Tigyi**[b], and **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

Abstract

Cyclic phosphatidic acid (CPA) is a naturally occurring analog of lysophosphatidic acid (LPA) in which the sn-2 hydroxy group forms a 5-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.

Keywords

lysophosphatidic acid; NPP2; autotaxin; GPR92; lysophospholipase D

Lysophosphatidic acid (LPA) is a pleiotrophic phospholipid growth factor with multiple roles in cancer metastasis and progression1.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

*Address correspondence to: Duane D. Miller, Ph.D. dmiller@uthsc.edu .

group forms a 5-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.8ATX has lysophospholipase D enzyme activity and is responsible for the hydrolysis of lysophophatidylcholine leading to the generation of LPA9;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 lymphoma17 and glioblastomamultiforme.18 ATX also plays an important role in the chemotherapeutic resistance of breast19 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. 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 hours of incubation at 37 °C using a FLEX stationII 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. However, **R-3-CCPA** was approximately 2 fold 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 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 acitvitaion 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 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 (Figure 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 5×10^4melanoma cells via the tail vein and divided randomly into 4 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 (Figure 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 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 stereo-selective activation by CCPA ligands.

Acknowledgments

This research was supported by NIH grant CA92160 (G.T.), Van Vleet Professorship (D.M.), Breast Cancer Research Foundation (N.P.) and Lpath Inc. (G.M.).

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

References and notes

1. Liu S, Umezu-Goto M, Murph M, Lu Y, Liu W, Zhang F, Yu S, Stephens LC, Cui X, Murrow G, Coombes K, Muller W, Hung MC, Perou CM, Lee AV, Fang X, Mills GB. Cancer Cell 2009;15:539. [PubMed: 19477432]
2. Parrill AL. Biochim.Biophys.Acta 2008;1781:540. [PubMed: 18501204]
3. Murakami-Murofushi K, Uchiyama A, Fujiwara Y, Kobayashi T, Kobayashi S, Mukai M, Murofushi H, Tigyi G. Biochim.Biophys.Acta 2002;1582:1. [PubMed: 12069804]
4. Fujiwara Y. Biochim.Biophys.Acta 2008;1781:519. [PubMed: 18554524]
5. Baker DL, Fujiwara Y, Pigg KR, Tsukahara R, Kobayashi S, Murofushi H, Uchiyama A, Murakami-Murofushi K, Koh E, Bandle RW, Byun HS, Bittman R, Fan D, Murph M, Mills GB, Tigyi G. J.Biol.Chem 2006;281:22786. [PubMed: 16782709]
6. Uchiyama A, Mukai M, Fujiwara Y, Kobayashi S, Kawai N, Murofushi H, Inoue M, Enoki S, Tanaka Y, Niki T, Kobayashi T, Tigyi G, Murakami-Murofushi K. Biochim.Biophys.Acta 2007;1771:103. [PubMed: 17123862]
7. Williams JR, Khandoga AL, Goyal P, Fells JI, Perygin DH, Siess W, Parrill AL, Tigyi G, Fujiwara Y. J.Biol.Chem 2009;284:17304. [PubMed: 19366702]
8. Stracke ML, Arestad A, Levine M, Krutzsch HC, Liotta LA. Melanoma Res 1995;5:203. [PubMed: 7496154]
9. Umezu-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J, Arai H. J.Cell Biol 2002;158:227. [PubMed: 12119361]
10. Tokumura A, Majima E, Kariya Y, Tominaga K, Kogure K, Yasuda K, Fukuzawa K. J.Biol.Chem 2002;277:39436. [PubMed: 12176993]
11. Tsuda S, Okudaira S, Moriya-Ito K, Shimamoto C, Tanaka M, Aoki J, Arai H, Murakami-Murofushi K, Kobayashi T. J.Biol.Chem 2006;281:26081. [PubMed: 16837466]
12. Yang Y, Mou L, Liu N, Tsao MS. Am.J.Respir.Cell Mol.Biol 1999;21:216. [PubMed: 10423404]
13. Wu JM, Xu Y, Skill NJ, Sheng H, Zhao Z, Yu M, Saxena R, Maluccio MA. Mol.Cancer 2010;9:71. [PubMed: 20356387]
14. Vidot S, Witham J, Agarwal R, Greenhough S, Bamrah HS, Tigyi GJ, Kaye SB, Richardson A. Cell Signal 2010;22:926. [PubMed: 20100569]
15. Kehlen A, Englert N, Seifert A, Klonisch T, Dralle H, Langner J, Hoang-Vu C. Int.J.Cancer 2004;109:833. [PubMed: 15027116]

16. Nouh MA, Wu XX, Okazoe H, Tsunemori H, Haba R, Abou-Zeid AM, Saleem MD, Inui M, Sugimoto M, Aoki J, Kakehi Y. Cancer Sci 2009;100:1631. [PubMed: 19549252]
17. Masuda A, Nakamura K, Izutsu K, Igarashi K, Ohkawa R, Jona M, Higashi K, Yokota H, Okudaira S, Kishimoto T, Watanabe T, Koike Y, Ikeda H, Kozai Y, Kurokawa M, Aoki J, Yatomi Y. Br.J.Haematol 2008;143:60. [PubMed: 18710386]
18. Kishi Y, Okudaira S, Tanaka M, Hama K, Shida D, Kitayama J, Yamori T, Aoki J, Fujimaki T, Arai H. J.Biol.Chem 2006;281:17492. [PubMed: 16627485]
19. Samadi N, Gaetano C, Goping IS, Brindley DN. Oncogene 2009;28:1028. [PubMed: 19079345]
20. van Meeteren LA, Ruurs P, Christodoulou E, Goding JW, Takakusa H, Kikuchi K, Perrakis A, Nagano T, Moolenaar WH. J.Biol.Chem 2005;280:21155. [PubMed: 15769751]
21. Ferguson CG, Bigman CS, Richardson RD, van Meeteren LA, Moolenaar WH, Prestwich GD. Org.Lett 2006;8:2023. [PubMed: 16671772]
22. Hoeglund AB, Bostic HE, Howard AL, Wanjala IW, Best MD, Baker DL, Parrill AL. J.Med.Chem 2010;53:1056. [PubMed: 20041668]
23. North EJ, Osborne DA, Bridson PK, Baker DL, Parrill AL. Bioorg.Med.Chem 2009;17:3433. [PubMed: 19345587]
24. North EJ, Howard AL, Wanjala IW, Pham TC, Baker DL, Parrill AL. J.Med.Chem 2010;53:3095. [PubMed: 20349977]
25. Yokoyama K, Baker DL, Virag T, Liliom K, Byun HS, Tigyi G, Bittman R. Biochim.Biophys.Acta 2002;1582:295. [PubMed: 12069841]
26. Durgam GG, Virag T, Walker MD, Tsukahara R, Yasuda S, Liliom K, van Meeteren LA, Moolenaar WH, Wilke N, Siess W, Tigyi G, Miller DD. J.Med.Chem 2005;48:4919. [PubMed: 16033271]

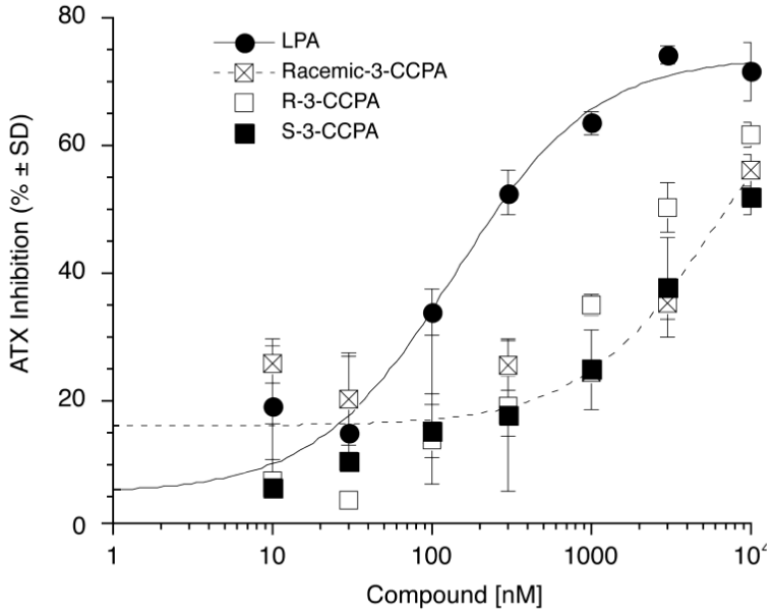


Figure 1.
Dose response relationship of ATX inhibition by LPA, R-3-CCPA, S-3-CCPA and Racemic-3-CCPA analogs.

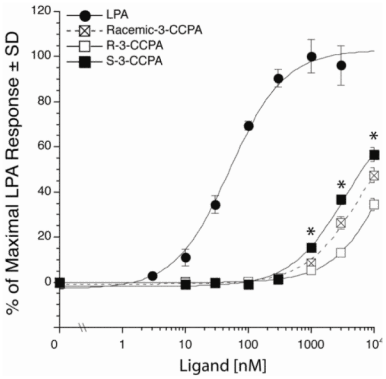


Figure 2.
Dose-response relationship of LPA_5 mediated

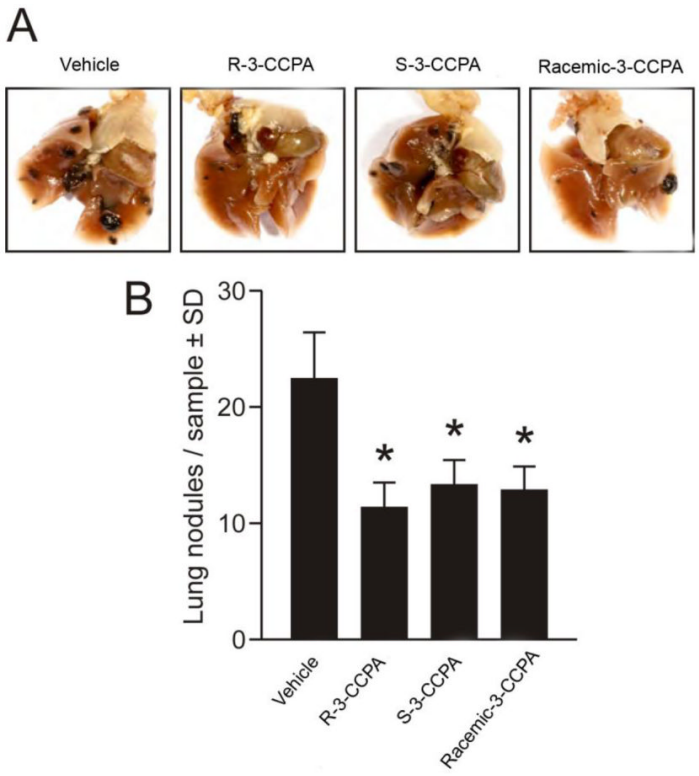
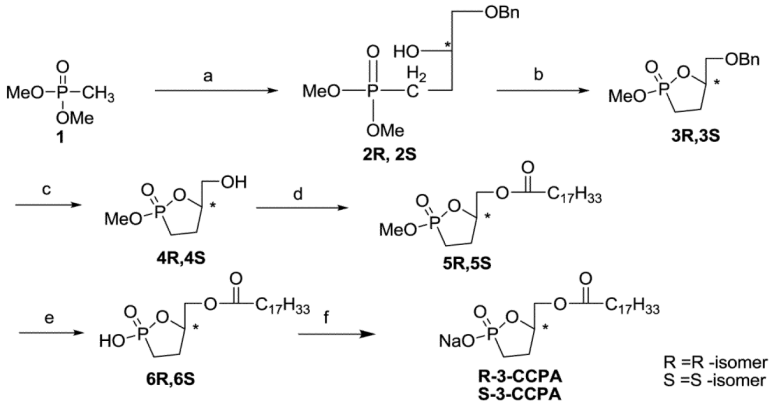


Figure 3. Lack of stereoselectivityin lung metastasis of B16-F10 melanoma cells by R-3-CCPA, S-3-CCPA and Racemic -3-CCPA analogs in a mouse model

A) Representative images show fixed intrathoracic organs including lung lobules with visiblenodules on the surfaces in black. Total numbers of nodules werereduced insamples treated with 7R, 7S 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.



Scheme 1.

(a) i) THF, n-BuLi (2.5M in hexane), −78°C, 0.5hr; ii) R-Benzyl glycidyl ether (R) / S-Benzyl glycidyl ether (S); iii) THF,BF_3OEt_2,−78°C, 2hr; iv) −20°C, 2hr, 68%; (b) PPTS, Toluene, Reflux, 5hr, 65% ; (c) H_2, $Pd(OH)_2$ / C,MeoH, 82.5%; (d) $C_{17}H_{33}COOH$, DMAP, DIC, DCM, 18hr, 78%; (e)TMSBr,CH_2Cl_2, 1hr, 53%; (f) 0.05M NaOH