Staurosporine
A new tool for studying phosphatidylserine trafficking

Kwang-Jin Cho, Jin-Hee Park and John F. Hancock*
Department of Integrative Biology and Pharmacology; The University of Texas Medical School at Houston; Houston, TX USA

Keywords: Ras GTPase, phosphatidylserine, lipid recycling, staurosporine, UCN-01

Ras proteins are small GTPases that regulate key pathways involved in cell growth, proliferation and differentiation. Ras proteins need to localize on the plasma membrane (PM) for its biological activity. Correct post-translational modification of the hypervariable region (HVR) at C-terminus of Ras is required for translocation to the inner leaflet of the PM. Constitutively active mutant K-Ras is expressed in pancreatic, lung and colon cancers, and is therefore a major clinical problem. Pharmacological agents that block K-Ras biological activity would have great clinical utility. Point mutations that prevent Ras post-translational processing block PM localization and abrogate all biological and oncogenic activity. Farnesyltransferase inhibitors (FTI), which block the first step of Ras post-translational modification should phenocopy this mode of Ras inhibition. However, in cells treated with FTIs K-Ras and N-Ras undergo alternative processing by geranylgeranyltransferase 1. Geranylgeranylated K-Ras and N-Ras localize normally to the PM, and are equipotent with farnesylated K- and N-Ras in transforming assays. Despite the clinical failure of FTIs for inhibiting K-Ras PM localization, the basic biological observation that preventing K-Ras PM localization abrogates transforming activity remains valid.

In our recent study, we developed and utilized a high content assay to identify compounds that inhibit PM localization of K-Ras and that may thereby inhibit its biological activity. We screened a microbial extracts library composed of ~10,000 metabolites, and identified 21 extracts that mislocalized K-Ras and/or H-Ras from the PM. Two extracts that displaced both K- and H-Ras from the PM contained staurosporines and analogs; 7-oxostaurosporine (OSS), UCN-01 and UCN-02. We subsequently showed that staurosporines are more active against K-Ras than H-Ras. In staurosporine-treated cells, K-Ras is translocated to the ER, early endosomes, late endosomes/lysosomes and mitochondria, while H-Ras is redistributed predominantly to the Golgi. Although staurosporines are protein kinase C inhibitors and can induce apoptosis at high concentrations, we showed that the Ras mislocalization mechanism is independent of protein kinase C inhibition and induction of apoptosis.

Phosphatidylserine (PS) is an anionic phospholipid that is asymmetrically concentrated on the inner leaflet of the PM, and confers electrostatic potential to the PM. Using a PS binding probe, we showed that OSS disrupts the subcellular distribution of PS without externalizing PS to the outer leaflet of the PM, indicating OSS does not inhibit PM flippase nor activate scramblases. In addition, OSS treatment decreased not only the total amount of K-RasG12V and PS on the inner leaflet of the PM but also the nanoclustering of K-RasG12V and PS on the PM (Fig. 1A and B). OSS did not change H-RasG12V nanoclustering but did significantly reduce the amount of H-RasG12V associating with the PM (Fig. 1C). These are important observations, since perturbation of Ras nanocluster organization dysregulates Ras-mediated cellular signaling. Consistent with the nanoclustering and PM localization data, staurosporine treatment significantly decreased cell proliferation and MAPK signaling in K-Ras-transformed cells. Taken together, these and other data suggest that PS provides membrane

*Correspondence to: John F. Hancock; Email: john.f.hancock@uth.tmc.edu
Submitted: 03/22/13; Revised: 04/18/13; Accepted: 04/19/13
Citation: Cho K-J, Park J-H, Hancock JF. Staurosporine: A new tool for studying phosphatidylserine trafficking. Commun Integr Biol 2013; 6: e24746; http://dx.doi.org/10.4161/cib.24746
than immediately before PS addition. We interpret these results as indicating that staurosporines target endosomal sorting/recycling of PS. When exogenous PS reaches the inner leaflet of the PM, the increased PS concentration on the inner leaflet corrects PM binding of K-Ras on a time scale that matches PS delivery from the outer to the inner leaflet. PS then undergoes normal endocytic recycling such that in the absence of staurosporines, internalized PS is recycled back to the PM by vesicular pathways. However, when staurosporines continuously block the sorting/recycling of PS back to the PM, the internalized PS is instead redistributed to endosomes. Consequently, PS concentration on endomembranes now is greater than before the PS supplementation, accounting for the observed enhancement of K-Ras mislocalization in OSS-treated cells supplemented with exogenous PS.

Clinical studies involving UCN-01 (7-hydroxystaurosporine) have been completed or are currently active for a wide range of tumors. Although UCN-01 inhibits protein kinases and dysregulates cell cycling, the exact mechanism of its anti-tumor activity is still unclear. Our study suggests a new mechanism that merits further investigation. A review of the study designs for UCN-01 would also be appropriate to see if an adequate sample of K-Ras-positive tumors was included. The identification of additional compounds that target PS lipid trafficking is now required to validate whether reducing PM PS content is a viable strategy to therapeutically modify K-Ras signaling.

In summary, staurosporines significantly decrease the level of PS on the inner leaflet of the PM, and mislocalize Ras proteins from the PM. PS depletion on the PM results in a reduced nanoclustering of K-RasG12V, suggesting that PS is required for both PM binding of K-Ras as well as the maintenance of K-RasG12V nanoscale spatial organization. Staurosporines disrupt the endosomal recycling of PS. The exact molecular target of staurosporine in PS recycling process is currently unknown, but whatever the mechanism staurosporine is a new pharmacological tool to study the cellular trafficking of PS.

**Materials and Methods**

*EM and spatial mapping of basal plasma membranes in polarized epithelial cells.* Basal sheets of MDCK cells transiently expressing mGFP-H-RasG12V were prepared exactly as described. The prepared basal PM sheets on gold EM grids were washed and fixed, and the cytosolic leaflet was labeled with anti-GFP antibody conjugated to 4.5 nm-gold particles. Spatial mapping of the gold-labeled Lact-C2 (A), K-RasG12V (C) or H-RasG12V (E) were performed. The L(r) curve is weighted mean K-function (n ≥ 15), where values above the 99% confidence interval (C.I.) for a random pattern indicate clustering at that value of r. Significant differences between the L(r) curves of OSS-treated and control cells were analyzed using bootstrap tests (*, p < 0.05; **, p < 0.01; ***, p < 0.001). The graphs show the mean number of gold particles/μm² (± S.E.M). Differences between OSS-treated and control cells were assessed using one-way ANOVA tests. Significant differences are indicated (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

*Disclosure of Potential Conflicts of Interest*

No potential conflicts of interest were disclosed.
References

1. Hancock JF, Paterson H, Marshall CJ. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. Cell 1990; 63:133-9; PMID:2208277; http://dx.doi.org/10.1016/0092-8674(90)90294-O

2. Hancock JF, Magee AI, Childs JE, Marshall CJ. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell 1989; 57:1167-77; PMID:2661017; http://dx.doi.org/10.1016/0092-8674(89)90054-8

3. Hancock JF. Ras proteins: different signals from different locations. Nat Rev Mol Cell Biol 2003; 4:373-84; PMID:12728271; http://dx.doi.org/10.1038/nrm1195

4. Prior IA, Lewis PD, Matros C. A comprehensive survey of Ras mutations in cancer. Cancer Res 2012; 72:2457-67; PMID:22589270; http://dx.doi.org/10.1158/0008-5472.CAN-11-2612

5. Baines AT, Xu D, Der CJ. Inhibition of Ras for cancer treatment: the search continues. Future Med Chem 2012; 7:905-14; PMID:22560614; http://dx.doi.org/10.1038/ncb1615

6. Willumsen BM, Christensen A, Hubbert NL, Papageorge AG, Lowy DR. The p21 ras C-terminus is required for transformation and membrane association. Nature 1984; 310:583-6; PMID:2661017; http://dx.doi.org/10.1016/0092-8674(89)90054-8

7. Whynne DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, et al. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem 1997; 272:14459-64; PMID:9162087; http://dx.doi.org/10.1074/jbc.272.22.14459

8. Cox AD, Hisaka MM, Buss JE, Der CJ. Specific isoprenoid modification is required for function of normal, but not oncogenic, Ras protein. Mol Cell Biol 1992; 12:2606-15; PMID:1375523

9. Cho KJ, Park JH, Piggot AM, Salim AA, Gore AA, Par ton RG, et al. Staurosporine disrupt phosphorylserine trafficking and localize Ras proteins. J Biol Chem 2012; 287:43573-84; PMID:23124205; http://dx.doi.org/10.1074/jbc.M111.242457

10. Leventis PA, Grinstein S. The distribution and function of phosphorylserine in cellular membranes. Annu Rev Biophys 2010; 39:407-27; PMID:20192774; http://dx.doi.org/10.1146/annurev.biophys.093008.131234

11. Tian T, Harding A, Inder K, Plowman S, Par ton RG, Hancock JF. Plasma membrane nanoswitches generate high-fidelity Ras signal transduction. Nat Cell Biol 2007; 9:905-14; PMID:17618274; http://dx.doi.org/10.1038/ncb1615

12. Cho KJ, Kassai RS, Park JH, Chigurupati S, Heidorn SJ, van der Hoeven D, et al. Raf inhibitors target ras spatiotemporal dynamics.Curr Biol 2012; 22:945-55; PMID:22560614; http://dx.doi.org/10.1016/j.cub.2012.03.067

13. Plowman SJ, Muncke C, Par ton RG, Hancock JF. H-ras, K-ras, and inner plasma membrane raft proteins operate in nanoclusters with differential dependence on the actin cytoskeleton. Proc Natl Acad Sci USA 2005; 102:15500-5; PMID:16223883; http://dx.doi.org/10.1073/pnas.0504114102

14. Kay JG, Kovuvalo M, Ma X, Wohland T, Grinstein S. Phosphorylserine dynamics in cellular membranes. Mol Biol Cell 2012; 23:2196-212; PMID:22496416; http://dx.doi.org/10.1091/mbc.E11-11-0936

15. Hore S, Oza A, Winquist EW, Moore M, Chen EX, Brown S, et al. Phase I trial of UCN-01 in combination with toposisomerase in patients with advanced solid cancers: a Princess Margaret Hospital Phase II Consortium study. Ann Oncol 2006; 17:334-40; PMID:16284058; http://dx.doi.org/10.1093/annonc/mdj076

16. Sauvage EA, Arbuck SG, Messmann R, Headeler D, Bauer KS, Lush RM, et al. Phase I trial of 72-hour continuous infusion UCN-01 in patients with refractory neoplasms. J Clin Oncol 2001; 19:2319-33; PMID:11304786

17. Li T, Christensen SD, Frankel PH, Margolin KA, Agarwala SS, Liu T, et al. A phase II study of cell cycle inhibitor UCN-01 in patients with metastatic melanoma: a California Cancer Consortium trial. Invest New Drugs 2012; 30:741-8; PMID:20967484; http://dx.doi.org/10.1007/s10637-010-9562-8

18. Edelman MJ, Bauer KS Jr., Wu S, Smith R, Bisaccia S, Dancey J. Phase I and pharmacokinetic study of 7-hydroxystaurosporine and carboplatin in advanced solid tumors, Clin Cancer Res 2007; 13:2667-74; PMID:17473198; http://dx.doi.org/10.1158/1078-0432.CCR-06-1832

19. Marti GE, Sterler-Stevenson M, Grant ND, White T, Figg WD, Tohnya T, et al. Phase I trial of 7-hydroxystaurosporine and fludarabine phosphate: in vivo evidence of 7-hydroxystaurosporine induced apoptosis in chronic lymphocytic leukemia. Leuk Lymphoma 2011; 52:2284-92; PMID:21745173; http://dx.doi.org/10.3109/10428194.2011.589547

20. Prior IA, Muncke C, Par ton RG, Hancock JF. Direct visualization of Ras proteins in spatially distinct cell surface microdomains. J Cell Biol 2003; 160:165-70; PMID:12527752; http://dx.doi.org/10.1083/jcb.20020991

21. Hancock JF, Prior IA. Electron microscopic imaging of Ras signaling domains. Methods 2005; 37:165-70; PMID:16288888; http://dx.doi.org/10.1016/j.ymeth.2005.05.018