Third International Electronic Conference on Synthetic Organic Chemistry (ECSOC-3), www.mdpi.org/ecsoc-3.htm, September 1-30, 1999

[C0002]

LQ12, A Novel PKC Activator, Enhances sAPP Secretion in PC-12 Cells

Lixin Qiao,[a]* Rene Etcheberrigaray,[a] Alan P. Kozikowski[b] and Bryan Roth[c]

[a]NeuroLogic Inc., 9700 Great Seneca Highway, Rockville, MD 20850, USA
E-mail: qiaol@giccs.georgetown.edu
[b]Drug Discovery Program, Georgetown Institute for Cognitive and Computational Science, 3970 Reservoir Road, Washington, DC 20007, USA
[c]Deparment of Psychiatry, Biochemistry, and Neurosciences, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA.

Received: 20 August 1999 / Uploaded: 24 August 1999

INTRODUCTION

Research on cellular and molecular aspects of Alzheimer's disease over the last two decades has resulted in a significantly improved understanding of this disease. Particular achievements have occurred in the areas of molecular genetics and the biology of amyloid. This research has strengthened the notion that Ab is a central player in the disease process.[1-5] Despite the progress and the data accumulated, the understanding of the pathophysiological framework remains incipient. Consequently, rational drug design targeting specific pathophysiological events or chemical entities has seen little or slow progress. Additional difficulties arise from the fact that dynamic processes are difficult to assess in the AD brain as a number of signal transduction mechanisms are altered by postmortem events.[6] In addition, AD brain tissue usually becomes available after a long period of the disease, at an advanced pathological state. Therefore, significant effort has been devoted to alternative approaches.[6-10] A host of cellular and molecular changes ranging from nucleic acid defects to second messenger alterations have been described in peripheral cells of AD patients.[6-8,10,11] Some alterations have also been observed in neuronal animal cells and brain.[12-15] Of the many alterations, PKC seems uniquely situated in the cascade of events (normal and pathological) interacting with Ab, calcium homeostasis and ion channel function. Herein we propose a framework as an initial step for drug design targeting PKC.

PROTEIN KINASE C

Protein kinase C is a ubiquitous family of enzymes involved in a number of cellular processes including (but not limited to) growth and differentiation, ion channel regulation, neuronal plasticity and memory storage.[16-20] Some of the functions may be related to specific isozymes and /or their differential expression in different tissues and organs. PKC has also been the subject of attention in a wide range of pathological processes from cancer to nervous systems disorders.[16, 20-22] A number of reports have identified alterations in protein kinase C in brains and fibroblasts of AD patients. Reduced levels of the particulate fraction in AD brains as measured by radioactive phorbol ester binding and in vitro phosphorylation of histone H I was originally reported by Cole et al.[23] Phosphorylation of P86 was also found reduced in the cytosolic fraction.[23] Additional reports have indicated that in particular the bII isoform was significantly lower in the particulate fraction from AD hippocampal and cortical tissues.[24] The opposite was true for the cytosolic fraction from cortex. The isoforms bI and a were also reduced in the particulate fraction from hippocampus of AD patients.[24] Shimoama et al. confirmed a predominant involvement of the b isoform in AD brains.[25] A more recent report found attenuated PKC activity and translocation in AD brains.[26] However, the immunoreactivity (membranous fraction) of the a and g isozymes was found increased in the frontal cortex. This finding was attributed to reduced proteolytic activity and/or a compensatory response in AD.[26] Some authors have suggested that PKC alterations may be an early event in AD.[27] Levels of mRNA for the a isozyme were found somewhat reduced in AD brains, although there was enough overlap so the differences did not reach statistical significance.[28] Other reports have not found salient differences in brain distribution of PKC in AD compared to controls.[29,30] Studies in fibroblasts generally parallel those in brain tissues. Both immunoreactivity and protein phosphorylation were found reduced in familial and sporadic AD fibroblasts.[31] More recently it was reported that the phorbol ester binding affinity and the phosphorylating activity (histone) were lower in cells from AD patients.[32] Immunoblotting analyses

revealed that the a isoform was primarily affected in AD fibroblasts.[33,34]

PKC may also play a significant role in APP processing. APP is the precursor protein from which b-amyloid originates and it possesses phosphorylation.[1,35] PKC activators influence the type of secreted APP products. The APP ectodomain can be constitutively secreted by cleavage within the b-amyloid sequence (a-secretase) thus resulting in "non-amyloidogenic" processing. APP can also be internalized and degraded in an endosomal/lysosomal compartment generating fragments that contain the intact b-amyloid sequence, therefore resulting in potentially amyloidogenic (and possibly pathologic) processing.[1,34,36] It has been shown that PKC activation can increase the rate of the non-amyloidogenic processing in cellular models. In addition, some studies have directly shown a reduction of the secretion of the b-amyloid peptide after phorbol treatment or activation of PKC.[34,35,37,38] There are, however, two reports that show an increase in sAPP without changes in Ab.[39,40] These apparently contradictory results could be due to tissue specific differences. In addition, Savage et al.[41] reported a phorbol-induced *decrease* in Ab species without a noticeable *increase* in sAPP secretion in mouse brain. Therefore, the general finding of reduced PKC amounts and/or activity in AD (brain and fibroblasts) is consistent with the apparently normal regulatory role of PKC favoring non-amyloidogenic processing of APP. It is worth noting that some of the Italian cell lines that showed the K^+ channel defect and enhanced IP_3 mediated calcium releases were the same ones in which defective PKC activity and immunoreactivity was demonstrated.[32-34,42,43] Interestingly, in these AD fibroblasts the reduced PKC activity has been correlated with a reduced ability to secrete soluble APP.[34,43] We report herein our recently designed novel PKC activators that are capable of increasing soluble APP.

DESIGN CONCEPT OF PYRROLIDONE ANALOGS

On the basis of the X-ray structure of PKC CRD2 (cysteine-rich domain) in complex with phorbol 13-acetate,[44] we have determined how the high-affinity ligands ILV and the eight-membered ring benzolactam bind through a combination of molecular modeling and site-directed mutagenesis studies.[45] To both simplify and to rigidify the ILV structure, conceptually we considered linking C-9 and N-13, as these atoms are close to one another in ILV's twist conformation (Figure 1a), to arrive at the pyrrolidone derivatives **6a** (Figure 2). In order for this type of compound to interact efficiently with PKC, modeling studies revealed that the isopropyl and phenyl groups must be cis oriented and trans to the hydroxymethyl group. With this stereochemistry, the pyrrolidone is capable of engaging in the same hydrogen-bond network to PKC as identified for ILV (Figure 1). Its isopropyl group interacts with the side chain of Leu 24, thereby mimicking the isopropyl group of ILV. Also, its phenyl group is parallel to Pro 11, thus allowing for strong hydrophobic interactions. However, the important interaction of the N-methyl group of ILV with Pro 11 and Leu 20 is absent (the absence of this group in ILV results in a 100-fold reduction in potency[46]). In addition, the optimal water solubility values (log WS)[47] can be adjusted by introduction of an appropriate substituent. This side chain generally enhances a ligand's binding affinity through interaction with the lipid membrane.

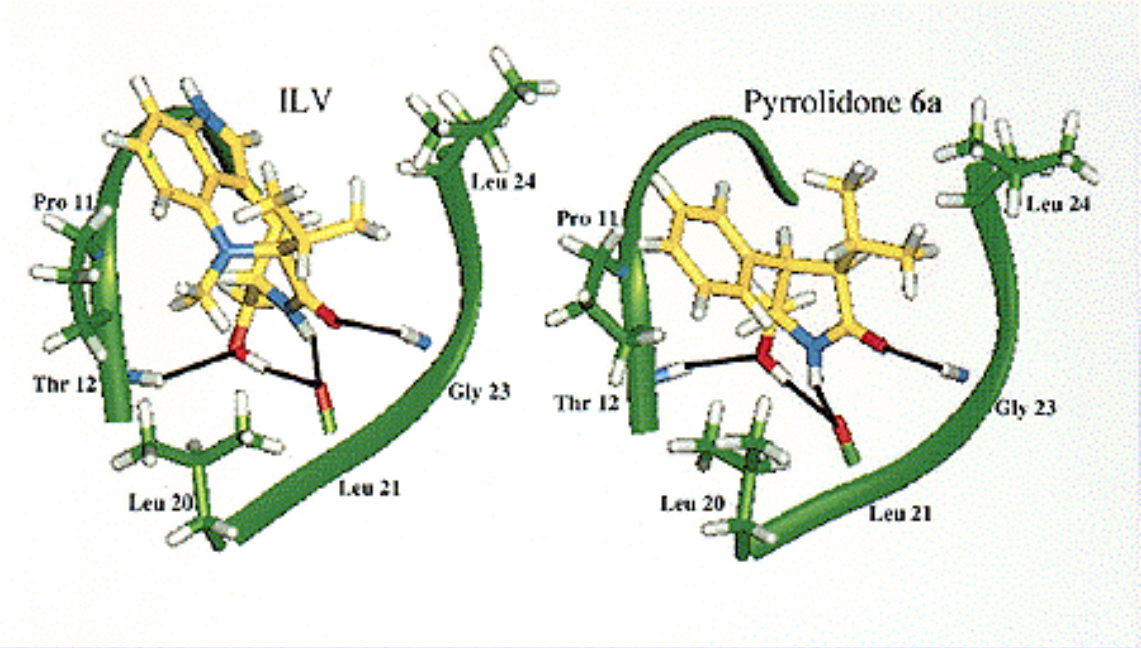


Figure 1. The overall features of the binding model for the ILV (left) and pyrrolidone derivative (right) in complex with PKCd CRD2.

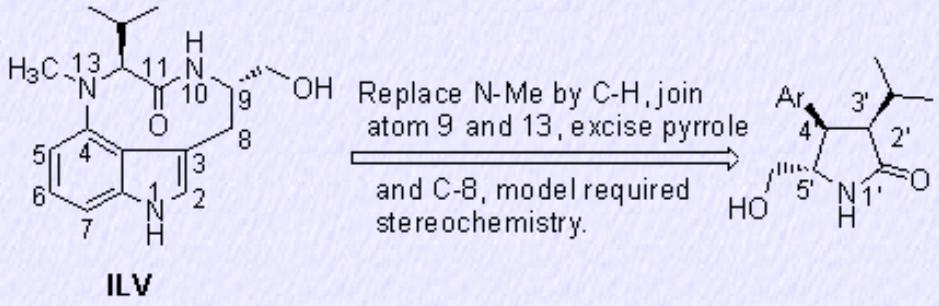
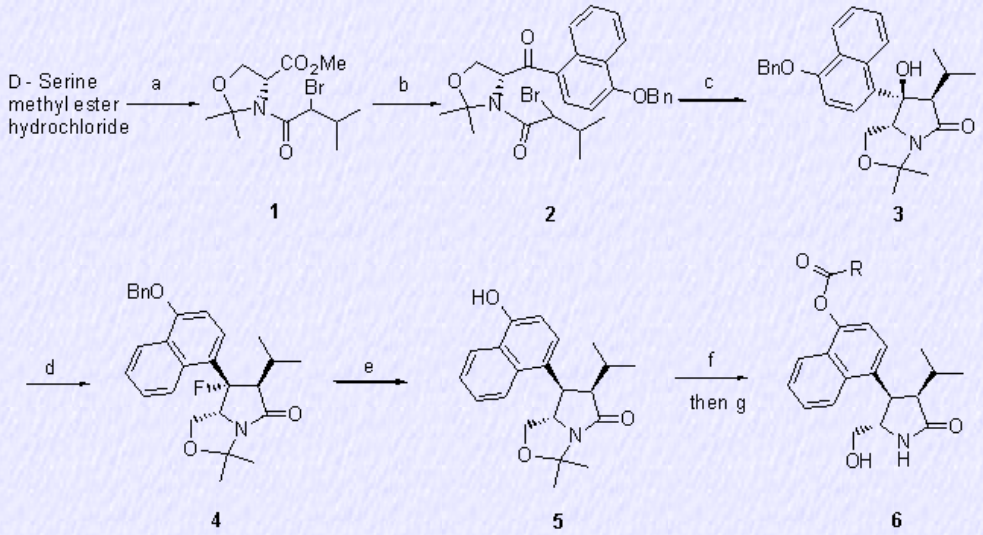


Figure 2. Strategy for design of pyrrolidone derivatives

CHEMISTRY

Based on our preliminary result of pyrrolidone **6a**,[48] molecular modeling suggested that the replacement of the phenyl in **6a** by an a-naphthyl can partially compensate for the absence of the interaction of ILV's N-methyl group with PKC. The synthesis of a series of substituted naphthyl pyrrolidones started from D-Serine. D-Serine methyl ester hydrochloride was first acylated with a-bromoisovaleryl chloride, followed by protection of the hydroxyl and amido groups with 2,2-dimethoxypropane. The resulting ester **1** was then reduced to aldehyde by DIBAL-H reduction, and treated with 4-benzyloxy-1-naphthylmagnesium bromide. The benzyloxy substituent allowed for the introducion of various side chains in later stages of this synthesis. Perruthenate-catalyzed oxidation of the resulting secondary alcohol furnished the ketone **2**. Subsequent SmI_2-mediated ring closure gave diastereoisomer **3**, whose stereochemistry was determined by ^1H NMR. With the exception of 4-aryl substituent, the ^1H NMR signals of compound **3** are similar to those of the simple naphthyl analog, the structure of which was previously determined by X-ray diffraction. Fluorination of the tertiary alcohol of compound **3** with DAST gave rise to compound **4**. Debenzylation and concurrent defluorination of **4** were successfully achieved by hydrogenation over 5% Pd/C in methanol affording compound **5**. In the ^1H NMR spectrum, the methine proton of the isopropyl group in **5** was shielded by the naphthyl ring to the extent of 0.8 ppm compared to that in **4**, supporting the cis-relationship of the isopropyl and naphthyl substituents. At this stage, esterification with various acyl chlorides was performed to introduce side chains for the purpose of obtaining optimum interaction with membrane. Final deprotection was achieved through transketalization with ethanedithiol in the presence of $BF_3 \cdot Et_2O$ to provide designed pyrrolidone analogs **6**.

Scheme 1



(a) 1. α-bromoisovaleryl chloride, Et_3N (2 equiv.), $CHCl_3$, 23 °C, 80%; 2. $Me_2C(OMe)_2$, PPTS (cat.), toluene, reflux, 77%; (b) 1. $(i\text{-}Bu)_2AlH$, CH_2Cl_2, -70 °C, 87%; 2. Grignard reagent (2 equiv.), THF, -78 to 0 °C, 55%; 3. NMO, TPAP (cat.), 4 Å M. S., CH_2Cl_2, 23 °C, 85%; (c) SmI_2 (3 equiv.), THF-HMPA, $FeCl_3$ (cat.), 23 °C, 80%; (d) DAST (2 equiv.), CH_2Cl_2, -78 °C, 80%; (e) H_2, MeOH, 5% Pd/C, 90%; (f) acyl chloride, pyridine, 23 °C, 80-93%; (g) 1,2-ethanedithiol (10 equiv.), $BF_3 \cdot Et_2O$ (2 equiv.), CH_2Cl_2, 23 °C, 60-85%.

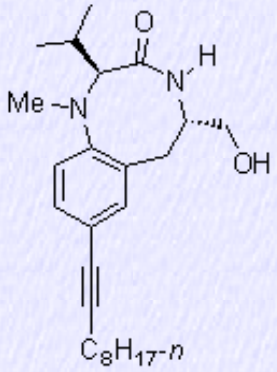
BIOLOGICAL EVALUATION

1. Binding affinity to recombinant PKCa

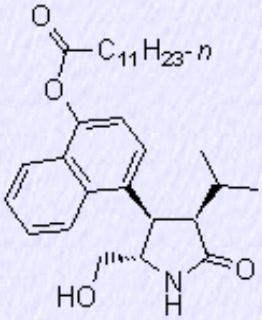
LQ12, a substituted naphthyl pyrrolidone, was tested for its ability to displace phorbol 12, 13-dibutyrate (PDBU) binding from recombinant PKCa. **BL**, an 8-membered benzolactam, was previously synthesized in our laboratory and shown to be a potent PKC activator. As is apparent from Table 1, **LQ12** is a fairly potent PKC activator. It is about four-fold less active than **BL**.

Table 1. *Ki* values for inhibition of [^{3}H]-PDBU binding to recombinant PKCa

	BL	**LQ12**
Ki ± SEM (nM)	23 ± 4	97 ± 35



BL



LQ12

Figure 3. Chemical structure of benzolactam (**BL**) and **LQ12**

2. sAPP secretion

Materials and methods were used as published[49] to evaluate sAPP secretion. Our previous results revealed that **BL** treatment induced elevations in sAPP among AD cell lines in a dose-dependent manner. Pretreatment with staurosporine, a potent PKC inhibitor, completely prevented the **BL**-induced sAPP secretion, supporting the involvement of PKC. As depicted in Figure 3,[49] **LQ12** significantly increased sAPP secretion in PC-12 cells and was found to be slightly less potent than **BL**. This effect on amyloid processing mirrors the binding results, further supporting the involvement of PKC.

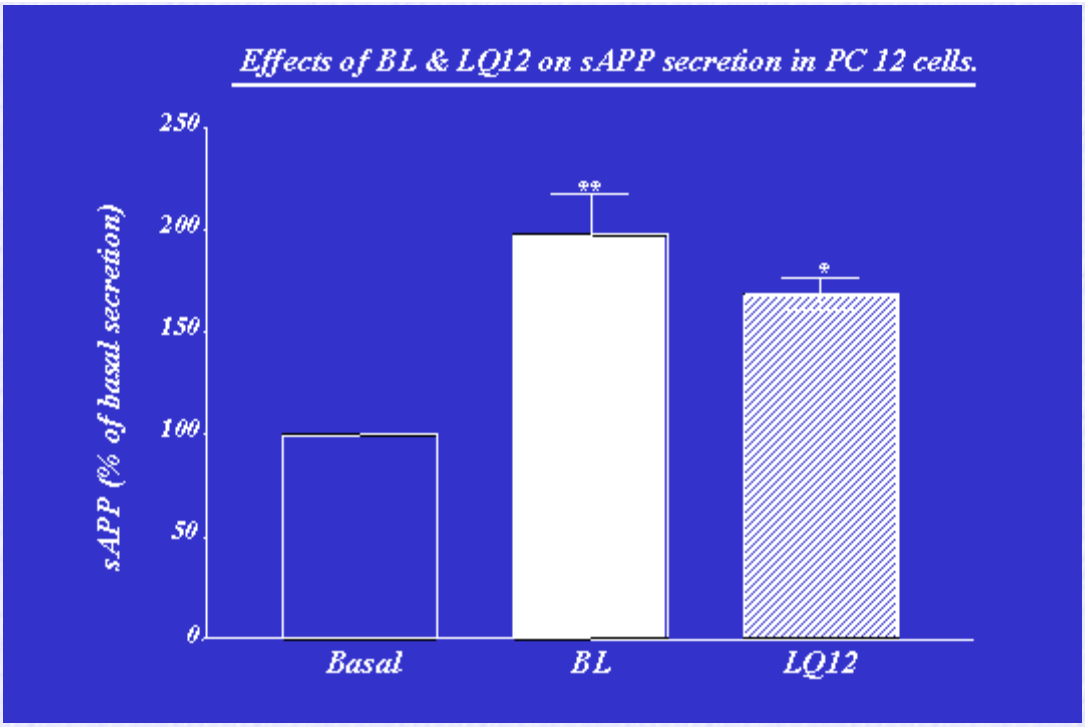


Figure 4. Effects of BL and LQ-12 on sAPP secretion in PC-12 cells.[49]

CONCLUSIONS

LQ12, a novel PKC activator, causes increased secretion of non-amyloidogenic sAPP in PC-12 cells. The elevated sAPP secretion may be also accompanied by a reduction of amyloidogenic fragments. The result further substantiates a key role for PKC in APP processing and, therefore, in AD pathophysiology. This study also suggests that PKC may be a useful target for preventing or slowing the pathophysiological process in AD. Furthermore, this novel compound offers the basis for drug design strategies targeted at PKC and APP processing that may significantly and beneficially alter the progression of this disease. Further studies aimed at optimizing the pyrrolidone structure so as to further maximize its activation of and therefore to maximize sAPP secretion are underway and will be released in due course.

ACKNOWLEDGEMENT

We thank the Department of Defense (DMAD 17-93-V-3018) and NIH grant CA79601 for the support of this research.

REFERENCES

1. Selkoe D.J. (1994) Normal and abnormal biology of the b**-amyloid precursor protein. *Annu. Rev. Neurosci.* 17, 489-517.**

2. Tanzi R. E., Kovacs D. M, Kim T., Moir R. D., Guenette S. Y., and Wasco W. (1996) The gene defects responsible for familial Alzheimer's disease. *Neurobiology of Disease* 3, 159-168.

3. Selkoe D. J. (1997) Alzheimer's disease: genotypes, phenotype and treatments. *Science* 275, 630-631.

4. Hardy J. (1997) Amyloid, the presenilins and Alzheimer's disease. *TINS* 20, 154-159.

5. Lendon C. L., Ashall F., and Goate A. M. (1997) Exploring the etiology of Alzheimer disease using molecular genetics. *JAMA* 277, 825-831.

6. Gibson G., Martins R., Blass J., and Gandy S. (1996) Altered oxidation and signal transduction systems in fibroblasts from Alzheimer patients, in *Life Sciences*, vol. 59, Muller W. E., and Gispen W. H., eds., Elsevier, Heidelberg, Germany, pp. 477-489.

7. Baker A. C., Ko L-W., and Blass J. P. (1988) Systemic manifestations of Alzheimer's disease. *Age* 11, 60-65.

8. Scott R. B. (1993) Extraneuronal manifestations of Alzheimer's disease. *JAGS* 41, 268-276.

9. Huang H-M., Martins R., Gandy S., Etcheberrigaray R., Ito E., Alkon D. L., Blass J., and Gibson G. (1994) Use of cultured fibroblasts in elucidating the pathophysiology and diagnosis of Alzheimer's disease. *Ann. N.Y. Acad. Sci.* 747, 225-243.

10. Gasparini L., Racchi M., Binetti G., Trabucchi M., Solerte B., Alkon D. L., Etcheberrigaray R., Gibson G., Blass J., Paoletti R., and Govoni S. (1997) Peripheral markers in testing pathophysiological hypotheses and diagnosing Alzheimer's disease. *FASEB J.* 12, 17-34.

11. Connolly G. P. (1998) Fibroblast models of neurological disorders: fluorescence measurement studies. *TiPS* 19, 171-177.

12. Good T. A., Smith D. O., and Murphy, R. M. (1996) b-amyloid peptide blocks the fast-inactivating K^+ current in rat hippocampal neurons. *Biophys. J.* 70, 296-304.

13. Cole G., Dobkins K. R., Hansen L. A., Terry R. D., and Saitoh T. (1988) Decreased levels of protein kinase C in Alzheimer brain. *Brain Res.* 452,165-174.

14. Masliah E., Cole G., Shimohada S., Hansen L., DeTeresa R., Terry R. D., Saitoh T. (1990) Differential involvement of protein kinase C isozymes in Alzheimer's disease. *J. Neurosci.* 10, 2113-2124 .

15. Shimohama S., Narita M. A., Matsushima H., Kimura J., Kamejama M., Hagiwara M., Hidaka H., and Taniguchi T. (1993) Assessment of protein kinase C isozymes by two -site enzyme immunoassay in human brains and changes in Alzheimer's disease. *Neurology* 43, 1407-1413 .

16. Lee-Way J., and Saitoh T. (1995) Changes in protein kinases in brain aging and Alzheimer's disease. *Drugs and Aging* 6, 136-149.

17. Olds J. L., and Alkon D. L. (1993) Protein kinase C: a nexus in the biochemical events that underlie associative learning. *Acta Neurobiol. Exp.* 53, 197-207.

18. Nishizuka Y. (1988) The molecular heterogeneity of protein kinase C and its implications in cellular regulation. *Nature* 334, 661-665.

19. Newton A.C. (1995) Protein kinase C: structure, function, and regulation. *J. Biol. Chem*. 270, 28495-28498.

20. Pascale A., Govoni S., and Battaini F. (1997) Age-related alterations of PKC, a key enzyme in memory processes: physiological and pathological examples. *Molecular Neurobiol.* In press.

21. O'Brian C.A., and Ward N. E. (1989) Biology of the protein kinase C family. *Cancer and Metastasis Reviews* 8, 199-214.

22. Nishino N., Kitamura N., Nakai T., Hashimoto T., and Tanaka C. (1989) Phorbol ester binding sites in human brain: characterization, regional distribution, age-correlation, and alterations in Parkinson's disease. *J. Molec. Neurosci*. 1, 19-26.

23. Cole G., Dobkins K. R., Hansen L. A., Terry R. D., and Saitoh T. (1988) Decreased levels of protein kinase C in Alzheimer brain. *Brain Res.* 452,165-174.

24. Masliah E., Cole G., Shimohada S., Hansen L., DeTeresa R., Terry R. D., Saitoh T. (1990) Differential involvement of protein kinase C isozymes in Alzheimer's disease. *J. Neurosci.* 10, 2113-2124 .

25. Shimohama S., Narita M. A., Matsushima H., Kimura J., Kamejama M., Hagiwara M., Hidaka H., and Taniguchi T. (1993) Assessment of protein kinase C isozymes by two -site enzyme immunoassay in human brains and changes in Alzheimer's disease. *Neurology* 43, 1407-1413 .

26. Wang H-Y., Pisano M. R., and Friedman E. (1994) Attenuated protein kinase C activity and translocation in Alzheimer's disease brain. *Neurobiology of Aging* 15, 293-298.

27. Masliah E., Cole G. M., Hansen L. A., Mallory M., Albright T., Terry R. D., and Saitoh T. (1991) Protein kinase C alteration is an early biochemical marker in Alzheimer's disease. *J. Neurosci.* 11, 2759-2767.

28. Chachin M., Shimohama S., Kunugi Y., and Taniguchi T. (1996) Assessment of protein kinase C mRNA levels in Alzheimer's disease brains. *Jpn. J. Pharmacol*. 71, 175-177.

29. Horsburg K., Deward D., Graham D., and McCulloch J. (1991) Autoradiographic imaging of [^{3} H] phorbol 12,13-dibutyrate binding to protein kinase C in Alzheimer's disease. *J. Neurochem.* 56, 1121-1129.

30. Clark E. A., Leach K. L., Trojanowski J. Q., and Lee V. M-Y. (1991) Characterization and distribution of the three major human protein kinase C isozymes (PKCa, PKCb, PKCg) of the central nervous system in normal and Alzheimer's disease brains. *Lab. Invest.* 64, 35-44.

31. Huynh T. V., Cole G., Katzman R., Huang K-P., and Saitoh T. (1989) Reduced protein kinase C immunoreactivity and altered protein phosphorylation in Alzheimer's disease fibroblasts. *Arch. Neurol.* 46, 1195-1199.

32. Govoni S., Bergamaschi S., Racchi M., Battaini F., Binetti G., Bianchetti A., Trabucchi M. (1993) Cytosol protein kinase C downregulation in fibroblasts from Alzheimer's disease patients. *Neurology* 43, 258-2586.

33. Racchi M., Wetsel W. C., Trabucchi M., Govoni S., Battaini F., Binetti G., Bianchetti A., and Bergamaschi S. (1994) Reduced protein kinase C immunoreactivity in fibroblasts from patients with Alzheimer's disease. *Neurology* 44, A164.

34. Racchi M., Wetsel W. C., Trabucchi M., Govoni S., Battaini F., Binetti G., Bianchetti A., and Bergamaschi S. (1994) Reduced protein kinase C immunoreactivity in fibroblasts from patients with Alzheimer's disease. *Neurology* 44, A164.

35. Gandy S., and Greengard P. (1994) Processing of Alzheimer Ab-amyloid precursor protein: cell biology, regulation, and role in Alzheimer disease. *Int. Rev. Neurobiol.* 36, 29-50.

36. Bhagavan S., Ibarreta D., Ma D., Kozikowski A. P., and Etcheberrigaray R. (1998) Restoration of TEA-induced calcium responses in fibroblasts from Alzheimer's disease patients by a PKC activator. *Neurobiology of Disease* 5, 177-187.

37. Desdouits F., Buxbaum J. D., Desdouits-Magnen J., Nairn A. C., and Greengard P. (1996) Amyloid b peptide formation in cell free preparations: Regulation by protein kinase C, calmodulin, and calcineurin. *J. Biol. Chem.* 271, 24670-24674.

38. Efhimiopoulos S., Punj S., Manopoulos V., Pangalos M., Wang G. P., Refolo L. M., and Robakis N. K. (1996) Intracellular cyclic AMP inhibits constitutive and phorbol ester-stimulated secretory cleavage of amyloid precursor protein. *J. Neurochem.* 67, 872-875.

39. Fuller S. J., Storey E., Li Q-X., Smith A. I., Beyreuther K., and Masters C. L. (1995) Intracellular Production of bA4 Amyloid of Alzheimer's disease: modulation by phosphoramidon and lack of coupling to the secretion of the amyloid precursor protein. *Biochemistry* 34, 8091-8098.

40. LeBlanc A. C., Koutroumanis M., and Goodyer C. G. (1998) Protein kinase C activation increases release of secreted amyloid precursor protein without decreasing Ab production in human primary neuron cultures. *Journal of Neuroscience* 18, 29097-2913.

41. Savage M. J., Trusko S. P., Howland D. S., Pinsker L. P., Mistretta S., Reaume A. G., Grrenberg B. D., Siman R., and Scott R. W. (1998) Turnover of amyloid b-protein in mouse brain and acute reduction of its level by phorbol ester. *The Journal of Neuroscience* 18, 1743-1752.

42. Hirashima N., Etcheberrigaray R., Bergamashi S., Racchi M., Battaini F., Binetti G., Govoni S., and Alkon D. L. (1996) Calcium responses in human fibroblasts: a diagnostic molecular profile for Alzheimer's disease. *Neurobiology of Aging* 17, 549-555.

43. Govoni S., Racchi M., Bergamaschi M., Trabucchi M., Battaini F., Bianchetti A. and Binetti G. (1996) Defective Protein Kinase C a leads to impaired secretion of soluble b-amyloid precursor protein from Alzheimer's disease fibroblasts. *Ann. N. Y. Acad. Sci.* 777, 332-337.

44. Zhang, G.; Kazanietz, M. G.; Blumberg, P. M.; Hurley J. H. *Cell* 1995, *81*, 917.

45. Kozikowski A. P., Wang S., Ma D., Yao J., Ahmad S., Glazer R. I., Bogi K., Acs P., Modarres S., Lewin N. E., and Blumberg P. M. (1997) Modeling, chemistry, and biology of the benzolactam analogues of ILV. 2. Identification of the binding site of the benzolactams in the CRD2 activator-binding domain of PKCd and discovery of an ILV analogue of improved isozyme selectivity. *J. Med. Chemistry* 40, 1316-1326.

46. Irie, I.; Okuno, S.; Kajiyama, S.; Koshimizu, K.; Nishino, H.; Iwashima, A. *Carcinogenesis* 1991, *12*, 1883.

47. Marquez, V.; Lee, J.; Sharma, R.; Teng, K.; Wang, S.; Lewin, N. E.; Bahador, A.; Kazanietz, M. G.; Blumberg, P. M. *Bioorg. Med. Chem. Lett.* 1994, *4*, 355.

48. Qiao, L.; Wang, S.; George, C.; Lewin, N. E.; Blumberg, P. M.; Kozikowski, A. P. *J. Am. Chem. Soc.* 1998, *120*, 6629.

49. Ibarreta D., Duchen M., Ma D., Qiao L., Kozikowski A. P., and Etcheberrigaray R. (1999) Benzolactam (BL) enhances sAPP secretion in fibroblasts and PC12 cells. *NeuroReport* 10, 1035-1040.

All comments on this poster should be sent by e-mail to (mailto:ecsoc@listserv.ariz ona.edu) ecsoc@listserv.arizona.edu with C0002 as the message subject of your e-mail.
