PKC activators PMA and AD 198: Functional Differences Due to Localization

Whitney Jo Barham

Department of Chemistry
Rhodes College
Memphis, Tennessee

2008

Submitted in partial fulfillment of the requirements for the
Bachelor of Science degree with Honors in Chemistry

This Honors paper by Whitney Jo Barham has been read and approved for Honors in Chemistry.

Dr. Loretta Jackson-Hayes
Faculty Sponsor

_____

Dr. David Y. Jeter
Second Reader

_____

Dr. Terry W. Hill
Extra-Departmental Reader

_____

Dr. Jon Russ
Department Chair

_____

Acknowledgements

I would like to acknowledge Dr. Leonard Lothstein and the pharmacology department at the University of Tennessee Health Science Center, Memphis, Tennessee. The entirety of this work was performed in the Lothstein lab, and would not have been possible without his mentorship. I would also like to acknowledge Luydmila Savranskaya (Mila) who taught me how to perform countless lab techniques, and Caroline Cook for learning along with me. May all glory be given to God, for in Him all things are possible.

Contents

List of Tables and Figures

Abstract

PKC activators PMA and AD 198:
Functional Differences Due to Localization

by

Whitney Jo Barham

Protein kinase C (PKC) is a family of serine/threonine kinases involved in numerous cell signaling pathways including the regulation of cell proliferation, differentiation and apoptosis. Because of this, the PKC family is a very attractive target for anti-cancer therapeutic drugs. In this project, two PKC binding agents were analyzed: the phorbol ester PMA (phorbol-12-myristate-13-acetate) and the anthracycline *N*-benzyladraimycin-14-valerate (AD 198). PMA and AD 198 are similar in their binding to PKC (the C1b domain), their cardio protective properties, and their ability to induce apoptosis in several mammalian cell lines when administered at certain doses. The two agents differ in their localization, among other things. PMA is found throughout the cell, whereas AD 198 is confined to the cytoplasm and can not enter the nucleus. PMA promotes differentiation in K562 chronic myeloid leukemia cells when administered at non-cytotoxic doses. Results indicate that AD 198 does not induce differentiation as PMA does due to its inability to translocate PKC **e** to the nuclear membrane and its inability to activate PKC a and its signaling pathway. These results suggest that differential cellular effects can be achieved by selective activation of PKC isoforms using PKC activating agents that localize differently within the same cell. Localizing the drug to the cytoplasm effectively limits its function, thereby creating a more targeted agent with more useful properties in drug therapy.

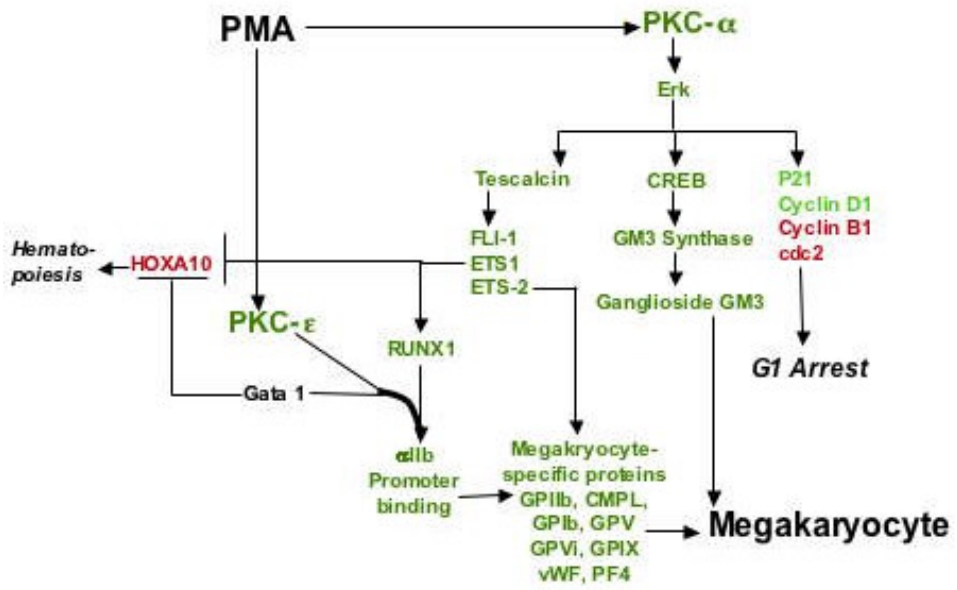
INTRODUCTION

Protein kinase C (PKC) is a family of serine/threonine kinases involved in numerous cell signaling pathways including the regulation of cell proliferation, differentiation and apoptosis. Because of this, the PKC family, which currently consists of some ten isozymes, is a very attractive target for anti-cancer therapeutic drugs.[1, 2] The general structure of PKC, like many kinases, consists of a catalytic domain and a regulatory domain joined by a hinge region. The catalytic domain is highly conserved and is the same in all isoforms. Therefore, the four subgroups of the PKC superfamily are divided based on differences in their regulatory domain. Alpha, beta, and gamma are considered the classic isoforms, and they require both Ca^{+2} and diacylglycerol (DAG) for activation. The novel subgroup, delta, epsilon, theta, and eta require only DAG for activation. The final two subgroups, atypical and the newly discovered PKC-related kinases (PRK's) require neither DAG nor Ca^{+2} for activation. In both the novel and classic subtypes, when the regulatory domain encounters its necessary substrate, PKC becomes activated and associates with the membrane in which the substrate is bound.

Within their regulatory region, both the classic and the novel PKC's contain a functional C1 domain which is responsible for their DAG binding ability. The C1 domain consists of two zinc fingers, a "top" and a "bottom" and is therefore divided into two subdomains, C1a and C1b. This same C1 domain allows the novel and classic PKC's to be bound by phorbol esters, which are non-hydrolysable DAG analogues. The most common phorbol ester is perhaps phorbol-12-myristate-13-acetate (PMA, also noted in some literature as 12-O-tetradecanoylphorbol-13-acetate or TPA), which has been shown to bind to the C1b domain of PKC to promote differentiation and apoptosis

through further PKC signaling.[3] Interestingly, only specific isoforms of PKC are involved in these signaling cascades due to the functional diversity among PKC isotypes.[4] In a broad context, the role of each individual isozyme in cellular signaling, and what each ultimately promotes (proliferation, differentiation, or apoptosis), has not been fully characterized. However, it is known that each isozyme is localized to a distinct area within the cell. This subcellular compartmentalization is indicative of unique functioning for each form,[5] and may account for their differential effects.

Activation of PKC isoforms alpha and epsilon have been implicated in PMA induced differentiation of leukemic hematopoietic cells [4, 6] (Figure 1). From previous findings, it is known that PMA induces differentiation in part through the activation of PKC a which in turn phosphorylates ERK (MAP kinase).[7] Phosphorylated ERK then activates a variety of things within the cell, including cAMP response binding elements (CREB), transcription factors that lead to the production of GM3 synthase (hST3Gal V), an enzyme which in turn synthesizes the ganglioside GM3. GM3 is known to induce megakaryocyte differentiation.[8] Furthermore, the phosphorylation of ERK by PKC a also leads to the upregulation of p21/waf, a cyclin dependent kinase inhibitor. Upregulation of p21 leads to G_1 cell cycle arrest, which is necessary for differentiation to occur.[9] PKC e is also involved in the induction of differentiation by PMA. It has been observed to synergize with both GATA 1 and RUNX1 transcription factors, binding to the aIIb promoter and causing the production of megakaryocyte specific proteins.[10]



Legend of Abbreviations

Upregulated
ERK – p42/44 Map Kinase
CREB – cAMP response element binding proteins
P21 – cyclin dependent kinase inhibitor 1A
FLI-1 – friend leukemia integration 1
ETS1 – V-ets erythroblastosis virus E26 oncogene homolog 1
ETS-2 – V-ets erythroblastosis virus E26 oncogene homolog 2
RUNX1 – runt-related transcription factor 1

Megakaryocyte specific proteins
cMpl – thrombopoietin ligand
GpIIb – glycoprotein IIb
GpIb –glycoprotein Ib
GPV- megakaryocytic antigen 4A
GPVi – glycoprotein VI
GPIX – glycoprotein IX
vWF - von Willebrand factor
PF4 – platelet factor 4

Downregulated
Cdc2 – cell division cycle 2; G1 to S and G2 to M
HOXA10 – homeobox A10

Figure 1. Known pathways of PMA induced differentiation in hematopoetic cells. PKC isoforms a and e are involved.

Mimicking DAG and binding to the C1b domain of PKC is not unique to the phorbol ester PMA. The novel anti-tumor agent N-benzyladriamycin-14-valerate (AD 198) is a PKC activator and also binds specifically to the C1b domain of PKC.[11] AD 198 is classified as an anthracycline drug, as it exhibits the typical structure of an anthracycline including a planar anthraquinone attached to a duanosamine sugar. Its parent compounds such as doxorubicin (DOX), which has been used clinically for over 25 years as an antitumor antibiotic, act by using their sugar structure to intercalate into DNA and inhibit topisomerase II activity. However, AD 198 is confined to the

cytoplasm and therefore uses a different mode of action than its parent anthracycline compounds. AD 198 has been shown to activate PKC d to trigger mitochondrial depolarization and apoptosis.[12]

One ongoing question is whether or not AD 198, like PMA, induces differentiation when administered to cells at non-cytotoxic doses. The two agents are somewhat similar. Due to their structural side-chain similarity (Figure 2), both PMA and AD 198 target the C1b domain of PKC. AD 198 promotes apoptosis in cells, as does PMA if given at a high enough dose. Both agents have also exhibited cardio-protective properties.[13, 14] However, in spite of these similarities, there are also known differences. AD 198 induced apoptosis is not affected by antiapoptotic Bcl2 proteins while PMA induced apoptosis is blocked by Bcl2's effects.[15] AD 198 is cytotoxic at a much lower concentration than PMA (Figure 3). Perhaps the most important difference is that the two drugs are localized differently in the cell. AD 198 remains only in the cytoplasm, whereas PMA is free to enter the nucleus. This could play a significant role in the ability to induce differentiation, as the association of the isoforms alpha and epsilon with the nucleus may be necessary to promote their downstream effects.

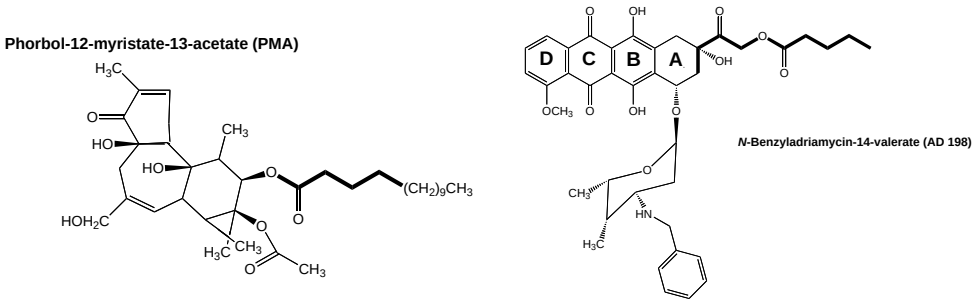


Figure 2. Structural comparison of PMA and AD 198.

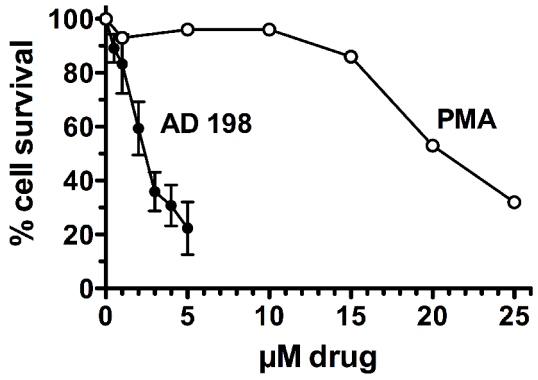


Figure 3. Cytotoxicity curve for AD 198 and PMA based on MTT assay

Since PMA induced differentiation has been well characterized in K562 chronic myelogenous leukemia cells, it provides an excellent model system in which to compare the actions of the two PKC activators and to assess AD 198's ability to induce differentiation. This study first asks if AD 198 induces differentiation at sub cytotoxic doses as PMA does. Furthermore, it seeks to explain AD 198's inability to induce differentiation if that is the case. Our hypothesis suggests there will be a difference in the two agents' function due to their different localization, further related to the differential localization of the PKC isoforms within the cell.

MATERIALS AND METHODS

Cell Culture and Reagents:

K562 chronic myelogenous leukemia cells were maintained in Dulbecco's modified Eagle meduium (DMEM) (Atlanta Biologicals, Norcross, Georgia, USA) supplemented with 10% FBS (Atlanta Biologicals). AD 198 hydrochloride salt was prepared in the Lothstein laboratory according to previously described procedures.[15] PMA was obtained from Promega Corporation (Madison, Wisconsin). Bryostatin was obtained from Calbiochem, EMD Biosciences, INC., (Darmstadt Germany). For use, all drugs were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in all drug treatments (2%) is not cytotoxic.

Fluorescent staining:

K562 cells were incubated with 1µM AD 198 for one hour, washed with phosphate buffered saline (PBS), and observed with an MVX10 MacroView (Olympus) fluorescent microscope. Nuclei were stained with H33342 DNA stain (1:100 stain/PBS ratio) after drug incubation in order to obtain figures with fluorescing nuclei.

RT-PCR

According to the manufacturer protocol, RNA was isolated with RNAstat-60 RNA isoloation agent (Tel-Test, Inc.). Ambion's (Austin, Texas) DNA-free DNase Treatment and Removal system was used according to manufacturer's protocol. 5 µg of each sample was combined with 0.2 µL reverse transcriptase (Promega), 0.2 µL Taq polymerase (New England BioLabs), 2 µL of the indicated primers (1 µL forward, 1 µL reverse), 25 µL of PreMix (100 mM Tris-HCl ph 3.3, 100 mM KCl, 3mM magnesium chloride, and 400 µM each dNTP), and volumes were brought-up to 50 µL with nuclease

free water. RT-PCR was then performed using the MJ Research Inc. (Watertown, Massachusetts) Minicycler, model PTC-150. See Table 1 for the settings used. Primer sequences were as previously described.[16,17]

Table 1. RT-PCR program settings

Step	Time	Temperature
1	30 min	37°C
2	1 min	95°C
3	30 sec	95°C
4	30 sec	*Reannealing temp (°C)
5	1 min	72°C
6	35 times to step 3	----
7	10 minutes	72°C
8	Up to 12 hours	4°C

*varied for each set of primers: GM3= 56, CD 61, Gfi-1b = 50, ß-actin = 54.

DNA was run on a 2% agarose gel and treated with ethidium bromide (Sigma Aldrich) stain. Results were visualized using UV light and captured digitally.

Flow-cytometry

After incubation with drug for 48 hours, cells were washed with cold PBS. Samples were pelleted and all excess PBS was removed. 1 mL absolute ethanol was added to each pellet and the sample was incubated at 4° C for one hour. Following this, the sample was again pelleted, and all excess ethanol was removed. Each sample was then dissolved in .5 mL of propidium iodide solution (3.8 mM sodium citrate and 50 µg/mL propidium iodide (Sigma P 4170) in PBS). Finally, 0.2 µL of RNAse was added to each sample. Samples were incubated at 4° C for 3 hours before analysis. Analysis was performed using the FACSAria Special Order System (BD Biosciences) located in the Molecular Science building at the Universtiy of Tennessee Health Science Center, Memphis, TN, according to manufacturer protocol.

Immunoblotting:

Immunoblot analysis was performed as described previously.[18] Briefly, total cell lysates were pelleted and washed with PBS. Samples were then dissolved in 200 µL of lysis buffer (12% 1.5M Tris buffer ph 8.8 and 28% of 20% SDS) and sonicated using a Microson ultrasonic cell disruptor. Protein determination was performed using the Bio-Rad Laboratories (Hercules, California) protein assay reagent system. Following protein determination of the samples, 50 µg of protein was resolved in 9% (ERK/PERK), 12% (p21), and 10% (PKC isoforms) polyacrylamide gels respectively and then transferred to nitrocellulose membrane (Bio-Rad Trans-Blot Transfer Medium). The membranes were blocked using 2% milk solution. The following primary antibodies were used at the dilutions noted: p21 $^{waf1/cip1}$ (Cell Signaling Technology) 1:1500 dilution; ß – tubulin (Cell Signaling Technology) 1:400 dilution; PKC a (Santa Cruz Biotechnology) 1:400 dilution; PKC e (Santa Cruz Biotechnolgy) 1:400 dilution; ERK (p44/42 MAP kinase antibody) (Cell Signaling technology) 1:800 dilution; PERK (phospho p44/42 MAP kinase antibody) (Cell Signaling Technology) 1:800 dilution. Membranes were incubated with primary antibody overnight. Goat anti-rabbit, horse radish peroxidase conjugated secondary antibody (Pierce, Rockford, Illinois) or goat anti-mouse, horse radish peroxidase conjugatged secondary antibody (Pierce) were used depending on the primary antibody type (polyclonal vs. monoclonal). A 1:400 dilution was used in both cases. Bands were visualized by Pierce SuperSignal West Pico reagents.

Cell fractionation:

After cells were exposed to drugs for times indicated, they were washed with cold PBS and re-suspended in 400 µL of homogenization buffer (250 mM sucrose, 0.5 mM

EGTA, 20 mM Hepes/KOH, pH 7.2). Inhibitors were added at the following dilutions: 1:400 DMSF, 1:400 leupeptin, 1:100 pepstatin, 1:200 aprotinin. Samples were incubated on ice for 20 minutes. Cells were lysed with a Dounce homognizer and the lysate was subjected to centrifugation at (800 G) for 5 minutes at 4° C. The resulting supernatant contained the cytoplasmic fraction. The pellet containing the nuclear portion was resuspended in lysis buffer (as described above in immunoblot procedure). Immunoblot was performed using the same procedure as above.

RESULTS

Fluorescent Microscopy to establish localization of AD 198

AD 198 is cytoplasmically localized within K562 cells, as previously described (Figure 4). [11] It is most concentrated within the membranous structures of the cytoplasm and in the perinuclear region with no detectable localization in the nucleus, the position of which is indicated by H3334 staining (Figure 4). PMA has been well characterized as an agent which can be found in both the cytoplasm and the nucleus of the cell.[19,20]

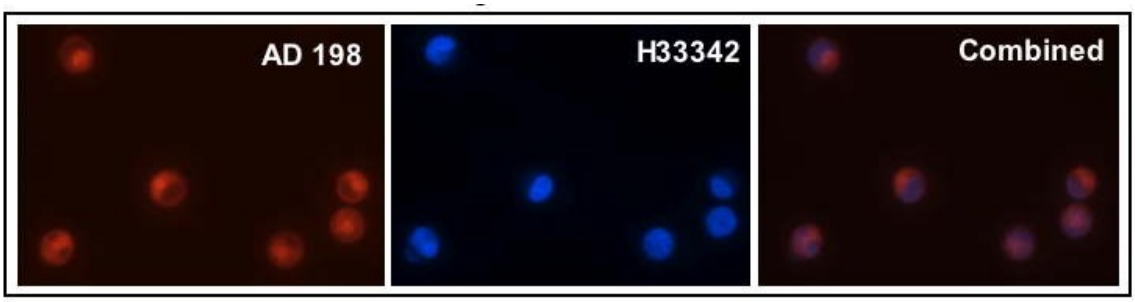


Figure 4. Fluorescent micrograph of AD 198 localization. From left to right: AD 198 (red autofluorescence only in cytoplasm; H33342 nuclear stain (blue fluorescence); combined fluorescence.

Morphologic changes and RT-PCR for cell specific antigens

Both PMA and AD 198 are cytotoxic in K562 (Figure 3). The maximum sub-cytotoxic dose of AD 198 was found to be 250 nm, corresponding to a similar amount of PMA cytotocity at 20 nm, a concentration previously shown to induce K562 differentiation.[21] These doses were used for all subsequent experiments.

Morphologic changes were observed after treatment with 20 nm PMA for 48 hours. Those cells treated with PMA showed characteristics associated with megakaryocyte differentiation including cell enlargement and multi-lobular nuclei (Figure 5A). Cells treated with sub-cytotoxic doses of AD 198 showed no signs of differentiation. To further assess the occurrence of megakaryocyte differentiation, RT-

PCR was used to probe for mRNA expression of megakaryocyte-specific antigens. PMA was found to induce the expression of CD61 (glycoprotein IIIa), a platelet specific antigen, signifying megakaryocyte differentiation. AD 198 did not induce this expression of CD61 (Figure 5B). Given that K562 cells are progenitors to both megakaryocyte and erythroid lineage, RT-PCR was also used to probe for the erythroid specific antigen Gfi-1B. Neither agent produced Gfi-1B mRNA expression. Therefore, neither agent induced erythroid differentiation.

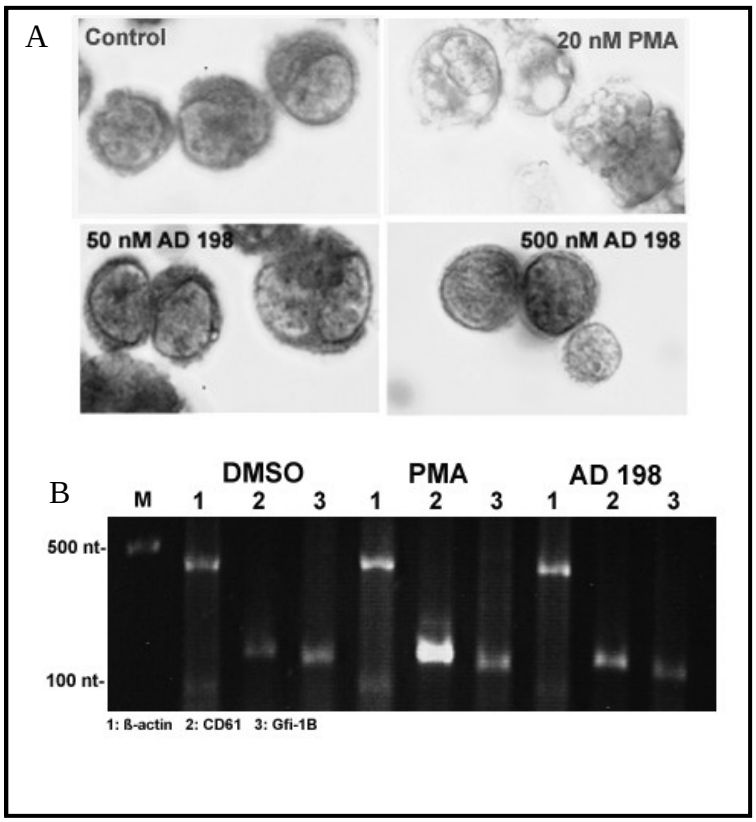


Figure 5. (a) K562 observed after treatment (b) RT-PCR for CD 61 and Gfi-1B using cells treated with DMSO (control), 20 nM PMA, and 250 nM AD 198.

Cell cycle analysis and p21 immunoblotting

Cells undergoing differentiation first become stalled in the G_1 phase of the cell cycle. This cell cycle arrest is caused by the action of certain cyclins. $P21^{waf/cip}$ is a kinase inhibitor that regulates these cyclins and promotes G_1 arrest. Both the upregultion

of p21 and G_1 cell cycle arrest are seen in cells treated with PMA. AD 198 does not cause G_1 cell cycle arrest (Figure 6A) nor does it induce the up-regulation of $p21^{waf/cip1}$ (Figure 6B) as PMA does. Bryostatin, a known PKC activator that does not promote differentiation, is able to block PMA induced differentiation in K562. AD 198, unlike bryostatin, is unable to block PMA induced differentiation as measured by p21 upregulation (Figure 6C). AD 198 acts as neither an antagonist nor an inhibitor of the differentiation pathway, and is mechanistically different from bryostatin.

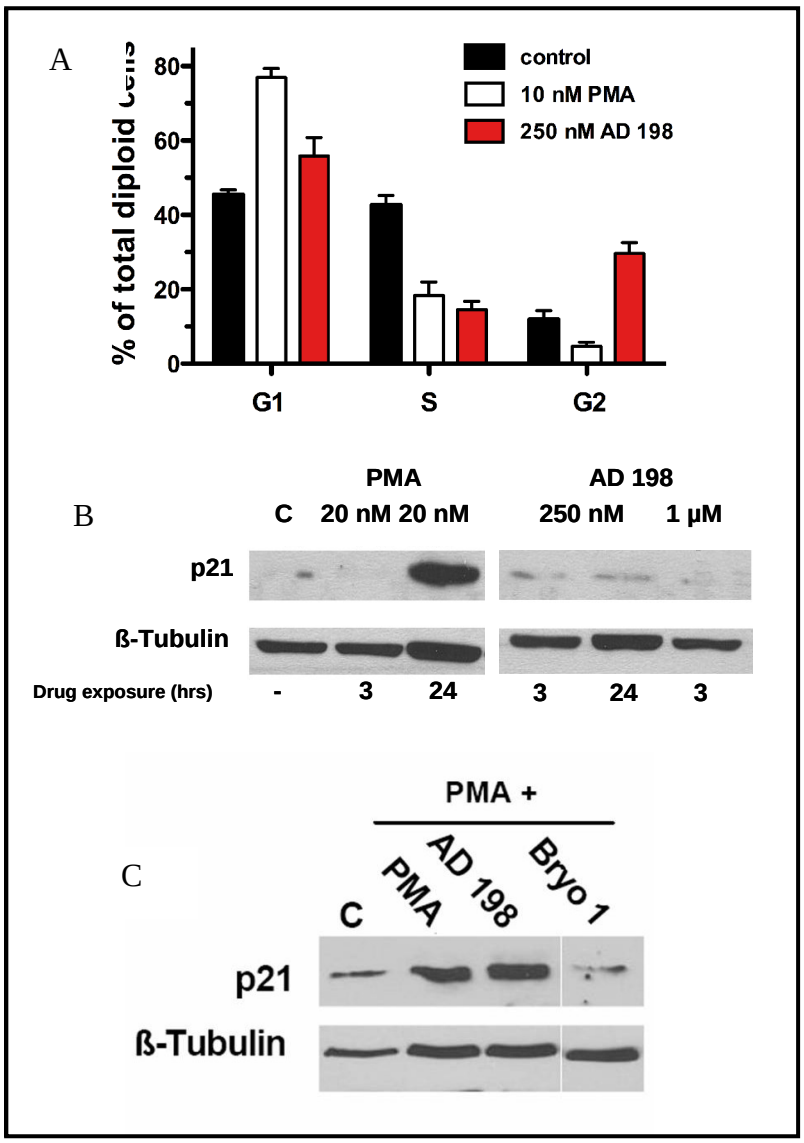


Figure 6. (a)Flow-cytometric analysis of exposed cells (b) p21 immunoblot after exposure to PMA and AD 198 (c) p21 immunoblot after pretreatment with 20 nm PMA, followed by exposure to 20 nm PMA, 250 nm AD 198, and bryostatin respectively.

PKC a signaling pathway

One arm of the observed pathway by which PMA induces differentiation begins with activation of PKC a. PMA activates PKC a by translocating it to the membrane. In contrast, AD 198 fails to translocate PKC a to the membrane (Figure 7A). Previous studies have suggested that PKC a activation by PMA results in sustained activation of ERK, via phosphorylation, and its translocation to the nucleus are necessary for differentiation.[22] PMA causes a sustained activation of ERK over a period of 72 hours (Figure 7B), whereas AD 198-mediated ERK activation fluctuates with a decrease at 48 hrs. In both PMA-and AD 198-treated cells phosphoylated ERK is found completely in the nuclear component, at the same levels, and identical to control (Figure 7C). This suggests that ERK translocation may not be essential for differentiation. Ganglioside GM3, which is implicated in differentiation is produced by GM3 synthase, whose expression is induced by PKC a. GM3 synthases expression is upregulated by PMA, but not by AD 198 (Figure 7D), thus further pointing to AD 198's inability to activate PKC a as a cause for its inability to induce differentiation.

PKC e signaling pathway

K562 cells were exposed to 250 nm AD 198 and 20 nm PMA, and samples were taken over a time course of 48 hours. Both agents induced the increased expression of PKC e (Figure 9). In the cells treated with 20 nm PMA, all of the PKC e was translocated to the nucleus. In contrast, AD 198 failed to translocate PKC e to the nucleus. This correlates with the inability of AD 198 to enter the nucleus.

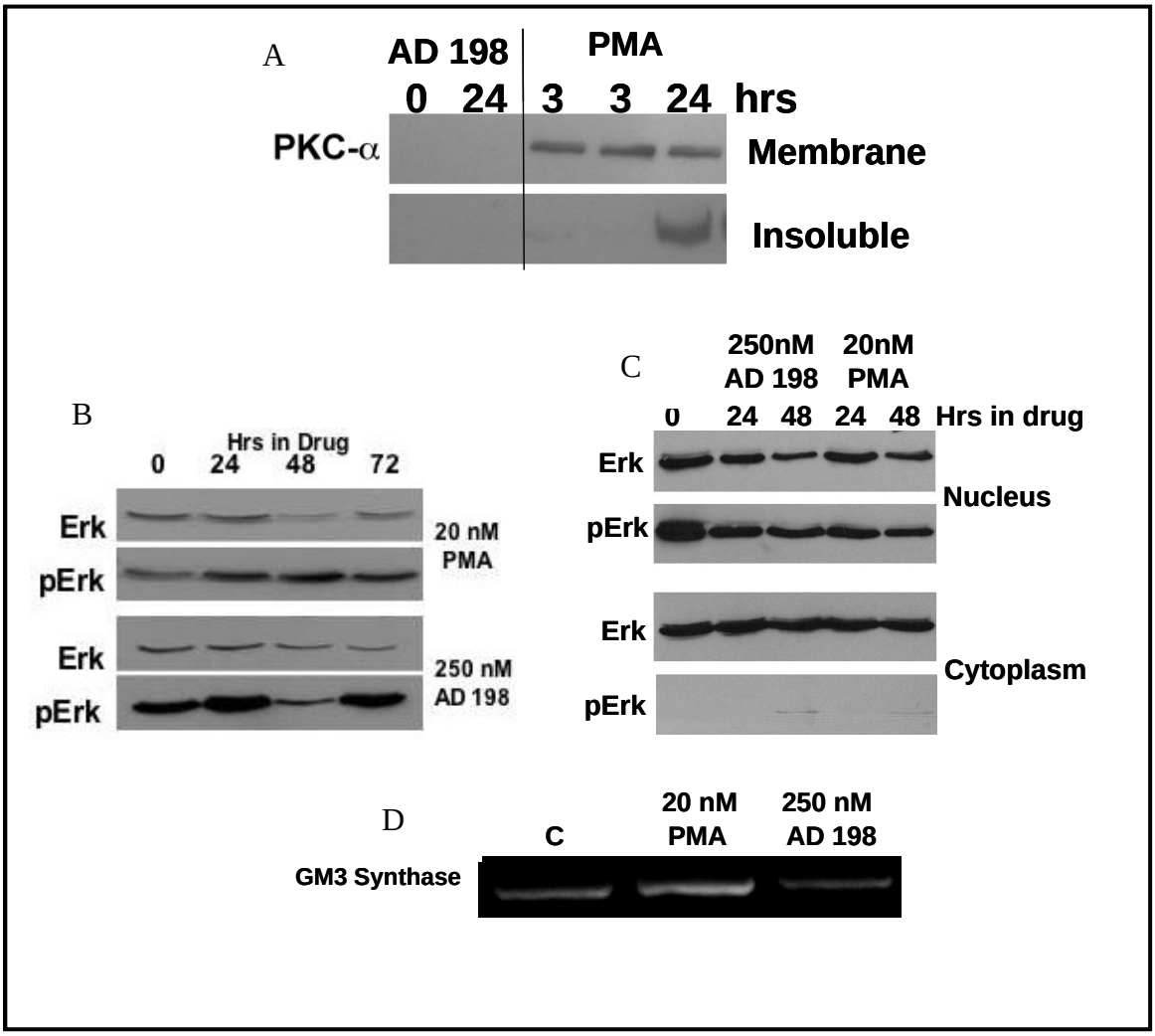


Figure 7. (a) Translocation of PKCa to membrane (b) ERK activation over 72 hour time course (c) Nuclear vs. cytoplasmic location of phosphorylated ERK in exposed cells (d) RT-PCR probe for GM3 synthase

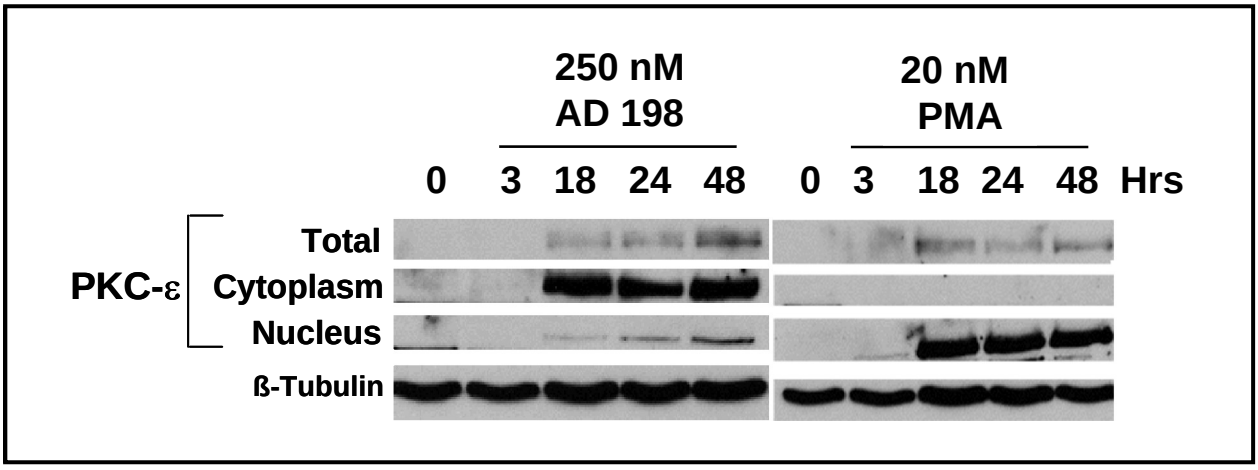


Figure 8. PKC e cytoplasmic vs. nuclear localization after exposure

DISCUSSION

In this study, we find that two PKC activators which bind to the same C1b domain can yield different cellular effects. Furthermore, the results also strongly suggest that this difference is due to the differing localization of the two drugs.

The first indication of a difference in effect was the morphologic changes seen in the PMA treated cells and the absence of these changes in those cells treated with AD 198. After probing for both CD61 and Gfi-1B mRNA's, it could be concluded that while neither agent promoted K562 to differentiate into erythroid cells, PMA induced megakaryocyte differentiation and AD 198 did not. This knowledge was important in characterizing the potential therapeutic agent AD 198, but determining *why* this molecule could not induce differentiation as PMA does had broader value and was therefore the focus of the remainder of this research.

Since G_1 cell cycle arrest is a necessary component of differentiation, flow cytometry was used to asses the state of cells treated with PMA and AD 198. These results showed a clear G_1 arrest promoted by PMA as compared to control cells. However, those cells treated with AD 198 showed only a slightly higher amount of G_1 arrest than control. A marked increase in G_2 cell cycle arrest was observed in those cells treated with AD 198. This could correlate with AD 198's cytotoxicity and ability to promote rapid apoptosis, as cells stalled in G_2 and at the G_2/M checkpoint have shown increased apoptosis in other studies.[23]

Closely related to G_1 cell cycle arrest is the cyclin dependent kinase inhibitor p21 which regulates the progression of the cell cycle at the G_1 stage. In PMA treated cells, p21 is upregulated, effectively leading to the G_1 cell cycle arrest observed in these cells.

In cells treated with AD 198, p21 was not upregulated, providing reason for the lack of G_1 cell cycle arrest seen in those samples. AD 198 also proved to behave mechanistically differently than the potent PKC activator bryostatin. While bryostatin is able to block PMA induced differentiation, AD 198 did not in our studies, and acted as neither an antagonist nor an inhibitor of PMA induced differentiation.

In addition to differences in cell cycle arrest and the promotion thereof, we hoped to identify other steps within the pathway at which AD 198 failed to act as PMA did. It was found that these points of divergence occurred at the beginning of the pathway, with AD 198's inability to translocate either of the PKC isoforms alpha and epsilon to the membrane. This is important because the effects of both isoforms are seen in the nucleus, as PMA acts to associate these isoforms with the nucleus and nuclear membrane, allowing them to further signal and promote megakaryocyte differentiation. Given AD 198's cytoplasmic localization, it would be unable to associate these isoforms with the nucleus in order to allow the promotion of differentiation.

Downstream targets in the pathway were also observed to be activated by PMA, but not by AD 198, indicating that no alternate pathway was being used by AD 198 to promote downstream effects. One such downstream effect was ERK (p42/44 MAPK) activation. A sustained activation of ERK has been noted as necessary for differentiation. While PMA promoted this sustained activation over a period of 72 hours, AD 198 showed fluctuation in its activation of ERK. Multiple trials were done to confirm the somewhat strange pattern of ERK activation promoted by AD 198, and each time there was initial activation followed by a sharp decrease at about 48 hours. Finally, there was a resurgence of activation at 72 hours. The reason for this up, down, and then up again

pattern has not yet been explained, but it remains clear that no sustained signal was induced by AD 198. Phosphorylated ERK (PERK) localization was also observed in the treated cells. Interestingly, cells treated with PMA and cells treated with AD 198 showed a similar pattern to control in this respect, with PERK localized entirely in the nucleus. Given the other results of this study, and with the knowledge that the ERK/PERK pathway is involved in numerous cell signaling events, we believe that the similar PERK localization could be a tangential effect, and not related to a similarity in the two drugs' actions.

The regulation of a downstream target of ERK, GM3 synthase, was observed in order to verify this concluision. GM3 synthase is the enzyme necessary to produce ganglioside GM3, which promotes the transcription of megakaryocyte specific proteins. GM3 synthase was upregulated in the PMA treated cells, but remained similar to control in those cells treated with AD 198. Therefore, this pathway showed clear activation by PMA, but no significant activation by AD 198.

While AD 198 does not activate PKC alpha and epsilon in K562 cells, it has been shown to activate PKC d. It triggers rapid apoptosis in proliferating cells by depolarizing mitochondria in a PKC d dependent[18] but calcium independent[12] manner. Therefore, AD 198's inability to activate PKC alpha and epsilon is not due to its inability to translocate *any* PKC isoform. Rather, AD 198 is more selective in its targets due to its cytoplasmic localization. It is able to activate PKC d and promote its downstream effects which occur in the cytoplasm.

Related to isoform localization, and its relationship to drug effects is the activation of PKC e in cardiomyocytes to provide cardioprotection. A common side-

effect, and dose limiting factor in clinical cancer therapeutics is cardiotoxicity. The role of PKC activation in cardioprotective signaling in cardiomyocytes has been well established [14], so agents that activate PKC have the potential to play a role in cardioprotection in addition to their anticancer effects. Phorbol esters, such as PMA, directly activate PKC e to achieve cardioprotection. Interestingly, AD 198 has also proven cardioprotective due to its ability to activate PKC e in cardiomyocytes. It is important to note that in these heart cells, PKC e's cardioprotective actions are promoted through its association with the plasma membrane, making both drugs capable of targeting this isoform. Its translocation to the nucleus is not necessary to gain cardio protective effects. This furthers the point that the localization of both drug and the isozyme are important in determining the effects of an agent.

To provide more support for these conclusions, the ideal experiment would be to modify AD 198 in order to allow it to enter the nucleus, while still maintaining the integrity of the drug, and determine if its ability to enter the nucleus then further allowed it to promote differentiation. In fact, this experiment was attempted in preliminary research through using AD 198 congeners, modified by one carbon on the acyl side chain (AD 442, 443, and 444) allowing them some nuclear localization. However, after investigation, these compounds proved to be only weak activators of PKC (approx. 20% of the activation seen by AD 198), thereby rendering them poor models and the results inconclusive. At this time, there seems to be no way to produce a legitimate model of AD 198 that both activates PKC at a reasonable level and is able to enter the nucleus.

Exploiting the PKC pathway for anticancer therapy is a technique used clinically today [24], and that is being further researched on many fronts. [25, 26] Agents such as PMA

are not ideal for clinical treatment for many reasons, not the least of which being that it is a possible tumor promoter and that its effects vary based on dosage. However, this research suggests that it should not be treated as the archetypal PKC activator and cause us to cease further investigation of other PKC activators for treatment. The results show that by limiting a drug's ability to enter the nucleus, the drug also becomes limited in its activation of PKC isoforms, and therefore becomes a much more attractive candidate for use.

By creating a drug with only a certain subset of effects, it is much more functionally specific. A "targeted agent" has been created, rather than a drug that has multiple effects based on dosage and cell environment. Not only is AD 198 targeted to the C1b domain of PKC, but also it is specifically targeted to those isoforms which carry out their signaling within the cytoplasm. This has important implications in drug design as we search for better, more effective ways to treat cancer.

Bibliography

1. Caponigro F, French, RC, Kaye SB. Protein kinase C: a worth while target for anticancer drugs? *Anticancer Drugs*1997*;* **8**:26-33.

2. Hoffman J. The potential for isozyme-selective modulation of protein kinase C. *FASEB J.* 1997; **11**:646-669.

3. Fujii T, García-Bermejo ML, Bernabó JL, Caamaño J, Ohba M, et al. Involvement of protein kinase C (PKC) in phorbol ester-induced apoptosis in LNCap prostate cancer cells. *Journal of Biological Chemistry* 2000; **275**:7574-7582.

4. Hocevar BA, Morrow DM, Tykocinski ML, Fields AP. Protein kinase C in human erythroleukemia cell proliferation and differentiation. *Journal of Cell Science* 1992; **101**: 671-679.

5. Goodnight JA, Mischak H, Kolch W, Mushinki JF. Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes. *Journal of Biological Chemistry* 1995; **270**:9991-10001.

6. Murray NR, Baumgardner GP, Burns DJ, Fields AP. Protein kinase C isotopes in human erythroleukemia (K562) cell proliferation and differentiation. *Journal of Biological Chemistry* 1993; **268**:15847-15853.

7. Herrera R, Hubbell S, Decker S, Petruzzelli L. A Role for the MEK/MAPK Pathway in PMA-Induced Cell Cycle Arrest: Modulation of Megakaryocytic Differentiation of K562 Cells. *Experimental Cell Research* 1998; **238**:407-414.

8. Choi HJ, Chung TW, Kang NY, Kim KS, Lee YC, Kim CH. Involvement of CREB in the transcriptional regulation of human GM3 synthase gene during megakaryocytoid differentiation of human leukemia K562 cells. *Biochemical and Biophysical Research Communications* 2004; **313**:142-147.

9. Steinman RA, Huang J, Yaroslavkiy B, Goff JP, Ball ED, Nguyen A. Regulation of p21(WAF1) Expression During Normal Myeloid Differentiation. ***Blood* 1998;** 91**:4531-4542.**

10. Racke FK, Mogass M, Khetawat R, Delehanty LL, Goldfarb AN. RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. *Blood* 2003; **101**:4333-4341.

11. Roaten, JB, Marcel GK, Caloca MJ, Bertics PJ, Lothstein L, Parrill AL, Israel M, Sweatman TW. Interaction of the novel anthracycline antitumor agent *N*-benzyladriamycin-14-valerate with the C1-regulatory domain of PKC: structural requirements, isoform specificity, and correlation with drug cytotoxicity. *Molecular Cancer Therapeutics* 2002; **1**:483-492.

12. Lothstein L, Savaranskaya L, Barrett CM, Israel M, Sweatman TW. *N*-benzyadriamycin-14-valerate (AD 198) activates protein kinase C-d holoenzyme to trigger mitochondrial depolarization and cytochrome c release independently of permeability transition pore opening and Ca2+ influx. *Anti-Cancer Drugs* 2006; **17**:495-502.

13. Hofmann PA, Israel M, Koseki Y, Laskin J, Gray J, Janik Aleksandra, Sweatman TW, Lothstein L. *N*-benzyladriamycin-14-valerate (AD 198): a non-cardiotoxic anthracycline that is cardioprotective through PKC-e activation. *The Journal of Pharmacology and Experimental Therapeutics* 2007; **323**:658-664.

14. Cohen MV, Baines CP, Downey JM. Ischemic preconditioning: from adenosine receptor to kATP channel. *Annual Review of Physiology* 2000; **62**:79-109.

15. Lothstein L, Rodrigues PJ, Sweatman TW, Israel M. Intracellular activity, distribution, and metabolism of *N*-benzyladriamycin-14-valerate (AD 198) are modulated by changes in the 14-O-acyl chain length. *Anticancer Drugs* 1998; **9**:58-66.

16. Osawa M, Yamaguchi T, Nakamura Y, Kaneko S, Onodera M, Sawada K, *et al.*, Erythroid expansion mediated by the Gfi-1B zinc finger protein: role in normal hematopoiesis. *Blood* 2002; **100**:2769–2777.

17. Jacques A, Herranz M, Legros L, Belhacene, Luciano L, Pages G, Hofman P, Auberger. Imatinib induces mitochondria-dependent apoptosis of the Bcr-Abl positive K562 cell line and its differentiation towards the erythroid lineage. *FASEB Journal* 2003; **14**:2160-2

18. Barrett CM, Roaten JB, Lewis FL, Sweatman TW, Israel M, Lothstein L. Drug-induced modulation of protein kinase C (PKC) correlates with circumvention of Bcl-2 mediated inhibition of apoptosis. *Molecular Cancer Therapeutics*. 2002; **1**:469-481.

19. Tran PL, Deugnier MA. Intracellular localization of 12-O-3-N-dansylamino TPA in C3H/10T1/2 mouse cell line. *Carcinogenesis* 1985; **6**:433-9.

20. Slater SJ, Stubbs CD. The use of fluorescent phorbol esters in studies of protein kinase cellular membrane intereactions. *Chem Phys Lipids* 2002; **116**:75-91.

21. Padilla PI, Wada A, Yahiro K, et al. Morphologic Differentiation of HL-60 Cells Is Associated with Appearance of RPTP ß and Induction of *Helicobacter pylori* VacA Sensitivity. *Journal of Biological Chemistry* 2000; **275**:15200-15206.

22. Racke FK, Lewandowska K, Goueli S, Goldfarb AN. Sustained Activation of the Extracellular Signal-regulated Kinase/Mitogen-activated Protein Kinase Pathway Is Required for Megakaryocytic Differentiation of K562 Cells. *Journal of Biological Chemistry* 1997; **272**: 23366-23370.

23. DiPaola P. To arrest or not to G_2-M cell cycle arrest. *Clinical Cancer Research* 2002; **8**:3311-3314.

24. Caponigro F, French RC, Kaye SB. Protein kinase C: a worthwhile target for anticancer drugs? *Anticancer Drugs* 1997; **1**:26-33.

25. Basu A,. The potential of protein kinase C as a target for anticancer treatement. *Pharmacol Ther* 1993; **59**:257-80.

26. Carter CA. Protein kinase C as a drug target: implications for drug or diet prevention and treatment of cancer. *Current Drug Targets* 2000; **1**:163-83.