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13. ABSTRACT (Maximum 200) The Protein Kinase C (PKC) family of kinases play a key role in the regulation of cell behavior. This final report for the proposal DAMD17-94-J-4166 describes our studies that set out to determine if particular isoforms of PKC are associated with a more malignant phenotype in human breast cancer. Expression of high levels of PKC-alpha and low levels of PKC-eta was associated with a more aggressive phenotype. Subsequent studies investigated the activity of two anti-PKC directed therapies in clinical trial.					
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FOREWORD

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Introduction

Metastasis is a complex, multi-step process that requires the coordinated expression of a number of properties by the tumor cells, which allow them to overcome the significant obstacles that lie between the primary tumor and the final metastatic site (1). These properties include altered cell-cell and cell-substratum adhesion, increased motility, the elaboration of proteases, altered growth control, and the ability to produce angiogenic factors. The mechanisms by which each of these processes are regulated are likely to be different. However, it is probable that all are modulated to some extent by the central signaling pathways used by cells. Indeed, many of the properties listed above have, for example, been shown in various model systems to be regulated by Protein Kinase-C (PKC)-mediated pathways; agents that modulate PKC have been shown to alter the rate of metastasis in some animal models. Furthermore, transfection of poorly invasive, non-metastatic cells with the PKC- α gene increased their metastatic potential.

There is an increasing body of evidence that different isoforms of PKC (a family of at least 11), are responsible for the regulation of different processes, and it was in the light of this that we originally hypothesize that metastatic behavior may in part result from: 1) the overexpression of specific isoforms of protein kinase C leading to increased activation of PKC responsive metastatic processes, and/or 2) an increase in the sensitivity of the cell to the signals that activate protein kinase C, resulting in increased kinase activity and stimulation of metastatic processes.

This Final report for DAMD 17-94-4166 will detail our work investigating the role of PKC in metastatic progression and the utility of anti-PKC therapeutic strategies. This project initially sought to determine the isoforms of PKC that are most closely associated with increased metastatic potential in breast tumors, and then after having identified several interesting associations, changed emphasis to examine the activity of two anti-PKC therapies in early clinical trials by way of correlative science studies.

Body

Background:

In spite of the great advances in screening techniques and public awareness that have been made over the past decade, resulting in the earlier detection of breast cancer, by the time many women know that they have a tumor and are receiving treatment, the disease will have already spread to other organs. The majority of the deaths from breast cancer are the result of these metastases. Therefore, learning about the process of metastasis and its regulation is of crucial importance in the fight against this disease.

The establishment of a metastasis is the result of a complex series of processes which seem to require that the tumor cell possess a number of specific abilities. Metastasizing cells must first change their adhesion properties to allow them to break away from the primary tumor. They must become more motile, and elaborate proteases to allow them to degrade and pass through the extra-cellular matrix and basement membranes and thereby gain entry to a lymphatic or blood vessel. Once in the circulatory system they must avoid destruction by immune surveillance or by the significant physical strains imposed by turbulence and shear forces in the circulatory system. Next, tumor cells arriving in the blood vessels of a target organ must attach to the endothelial cells that line the blood vessel, and somehow pass between them. After crossing another basement membrane, the cells must start to proliferate in the new site and recruit blood vessels into the growing tumor.

A considerable amount has been learned about the regulation of the various steps involved in this passage of a cancer cell from the primary tumor to the final metastatic site and the growth of the resultant metastasis. It is difficult, however, to determine from the literature which of these steps represent the key control points for invasion and metastasis. One common facet of all of the processes outlined above is, however evident from the literature, and that is that all of them have been reported to be regulated by protein kinase C mediated pathways: The expression of many of the cellular adhesion molecules implicated in these processes is regulated by PKC, including E-cadherin, CD44, and some integrin subunits. The adhesion of tumor cells to basement membrane components is inhibited by PKC inhibitors. The motility of cancer cells is stimulated by molecules that use PKC signaling pathways, and direct activation of PKC can stimulate motility. The expression of matrix degrading enzymes is stimulated by PKC activation. The adhesion of tumor cells to endothelial cells is enhanced by PKC activation. The retraction of endothelial cells in response to tumor cell binding is blocked by PKC inhibitors. Finally, protein kinase C is intimately involved in the control of the proliferation of all cell types including tumor cells and hence the growth of any metastasis.

What has also become more clear over the last few years is that many of the interactions between a cell and its physical environment, including those involved in the invasive process, can themselves cause changes in the activation and expression of PKC. For example, while it is known that the level of a number of integrin subunits is regulated by PKC, it has also been found that integrins on binding to their substrates can cause the activation of the tyrosine kinase pp125^{fak} (focal adhesion kinase) which has been shown to interact with intracellular signaling systems including those involving activation of PKC. In addition to this indirect evidence for the role of PKC in metastasis, there is also direct evidence, which will be discussed later, that activation of protein kinase C can stimulate metastasis.

There are at least 9 isoforms of this serine/threonine kinase, and undoubtedly more will be discovered. It is believed that the different isoforms of PKC may be responsible for the mediation of different PKC regulated effects, and there is evidence that some isoforms in particular are associated with malignant progression.

We have investigated the effects PKC activators and inhibitors on a number of the properties thought to be important in metastasis, and have found that in breast cancer cells they are dramatically stimulated by PKC activation. From these data and those in the literature we hypothesized that the acquisition of a metastatic phenotype may result in part from: 1) the overexpression of specific isoforms of protein kinase C, leading to increased activation of PKC responsive metastatic processes, and/or 2) an increase in the sensitivity of the cell to the signals that activate protein kinase C, resulting in increased kinase activity, and stimulation of metastatic processes.

Determination of PKC isoform levels in human breast cancer cell lines and correlation with invasive and metastatic potential.

To determine if there was a correlation between the level of particular PKC isoforms and metastatic behavior in human breast cancer cell lines, as we had hypothesized that there would be, we conducted northern and western blot studies to measure RNA and protein levels. The results from these data were correlated with data from *in vitro* determinations of the invasiveness and motility of the cells. We also compared these results with earlier experiments that we had conducted to determine the invasive and metastatic potential of the cells under study when grown as tumors in the nude mouse. Several interesting observations were made which subsequently were integrated into the design of clinical trials of two anti-PKC directed therapies. These studies have been reported in earlier annual reports, but as required are outlined here.

Determination of PKC levels in human breast cancer cell lines.

Provenance and Culture of Cells Used in the Study:

All of the cell lines examined were obtained from the Lombardi Cancer Center Tissue Culture Shared Resource as frozen vials and were grown in Improved Modified Eagles Medium (IMEM) supplemented with 10% Fetal Calf Serum (FCS). After thawing the cells were passaged at least twice before being plated at approximately 20% confluence prior to culture to approximately 70% confluence for use in experiments. The reason for this strategy is described below. Samples of all of the cell lines were frozen at the commencement of the study to provide an index stock of cells for subsequent experiments. All cells used were tested and shown to be free of mycoplasma contamination by the Tissue Culture Shared Resource.

Northern Blot Analysis:

Total cellular RNA was prepared from T75 flasks of cells grown to 70% confluence using standard methods (1). Aliquots (5 μ g) were fractionated through 1.2 % agarose gels containing 2.2M formaldehyde, and transferred to Hybond-N (Amersham, Arlington Heights IL). The uniformity of RNA transfer was verified by examining the ethidium-stained (0.5 μ g/ml) ribosomal RNA bands on the filters under UV illumination. The filters were then

hybridized using conditions previously described with riboprobes transcribed from plasmids containing cDNAs for the various PKC isoforms. After washing, the filters were exposed to Fuji-RX film at -70°C against intensifying screens, or analyzed with a phosphorimager (Molecular Dynamics Model 445 SI, Sunnyvale, CA). The filters were subsequently hybridized with a probe for 36B4, as a loading control. The expression of 36B4, which codes for a ribosomal protein, has been shown to be unchanged in these cells by treatment with TPA. Probes for the various PKC isoforms were either obtained from the ATCC or cloned by RTPCR from the RNA of cells that express them. Bands were subcloned into pGEM4z (Promega, Madison, WI) and subsequently used to generate riboprobes (1).

RNAse Protection Assay

RNAse protection assays were performed for some PKC isoforms using standard techniques on total RNA prepared from cells as described above (1). Briefly, 50 µg aliquots of RNA were hybridized with ³²P labeled anti-sense riboprobes for PKC isoforms and. The PKC probes were transcribed from approximately 300 bp fragments of the cDNAs subcloned into pGEM4z (Promega, Madison, WI). After digestion with RNAse A, proteinase K digestion and phenol chloroform extraction, the protected fragments were precipitated with ethanol, dissolved in loading buffer and analyzed by running them on a pre-cast 6% TBE urea gels (Novex, San Diego, CA). The gels were dried and the radioactive bands imaged and quantitated with a phosphorimager (Molecular Dynamics model 445 SI, Sunnyvale, CA).

Western Blot Analysis:

Western blot analysis of PKC levels was conducted using standard methods. Protein lysates were prepared from human breast cancer cell lines, grown as described above to 70% confluence. The cell monolayers were carefully washed free of contaminating serum proteins by rinsing twice with 25 ml of PBS. After draining for a short period and the aspiration of any remaining fluid, the monolayers were coated with 3 ml of a boiling lysis buffer which contained: 10mM Tris pH 7.5, 1% SDS, and to inhibit any phosphatase action: 5mM sodium pyrophosphate, 100uM Sodium vanadate, and 50mM sodium fluoride. After pipetting this solution over the cells for a few minutes to ensure the total dissolution of the monolayer and to start to reduce the viscosity of the solution by shearing the DNA, the lysates were transferred to 15 ml polypropylene tubes and placed in a boiling water bath for 10 minutes. This treatment ensures complete solubilization and further reduces the viscosity of the solution. After cooling, a sample of each lysate was removed for protein determination and the remainder diluted 1:1 with 2 X SDS PAGE loading buffer, aliquoted and frozen at -80C until analyzed. Protein levels were determined by the BCA assay per the manufactures instructions (Pierce, Rockford IL).

To determine the PKC isoform levels in the protein samples, equal amounts of protein (20ug) were fractionated through 10% SDS PAGE gels, and then transferred to nitrocellulose membranes using standard methods. Blocking of the non-specific binding sites on the membranes was conducted by several methods during the project as it was found that different batches of secondary antibody produced slightly different background problems. Internal standards were used to validate the results obtained using these different methods – the only differences were alterations in the level and pattern of secondary induced background bands. Blocking conditions were: blocking with 3% BSA in PBS with 0.1% Tween-20 (PBST) for 5 hours at room temperature or overnight at 4C, blocking in 5% Non-Fat Dried Milk (NFD) in

PBS or PBST at 4°C for 5 hours or overnight. The primary anti PKC antibodies came from a variety of sources. Antibodies against PKC α , β , δ , ϵ , θ , λ / ι μ and ζ came from Transduction Labs (Lexington, KY), antisera against PKC γ and η came from Santa Cruz Biotechnology Inc (Santa Cruz, CA). The antibodies were used at the dilutions recommended by these manufacturers in the blocking solution that had been used and the membranes were incubated in the antibodies at room temperature for 2 hours with gentle reciprocating agitation. After the primary antibody incubations the membranes were washed 5 times with PBST for 20 minutes and then exposed to either an anti-mouse (PKC α , β , δ , ϵ , θ , λ / ι and μ) or an anti-rabbit antibody (PKC γ and η) conjugated to horseradish peroxidase (HRP) at a 1:10,000 dilution (Bio-Rad) in the appropriate blocking buffer. Incubation was for 1 hour at room temperature after which the membranes were washed again 5 times with PBST for 20 minutes. Areas of immuno-reaction were then detected using ECL reagents (Amersham, Arlington Heights, IL), and X-ray film (Kodak XR-5). Several exposures were taken of each western blot to ensure that a good exposure for densitometry was obtained. Western-blot analysis was repeated at least 3 times for each lysate and results from subsequent lysate preparations were also examined.

Boyden Chamber Assays:

Motility and invasion assays were performed essentially as described before [2]. Assays were conducted using a 48-well micro Boyden chamber apparatus (Neuroprobe, Cabin John, MD). For motility assays, 80 x 25 mm polycarbonate filters (12 μ m pore; PVP free, Poretics, Livermore, CA) soaked in 0.01% gelatin (Sigma, St Louis, MO) were dried and placed in the apparatus separating the upper from the lower wells. The lower wells contained 25 μ l of fibroblast conditioned media prepared by incubating confluent monolayers of NIH 3T3 fibroblasts for 24 hours with IMEM containing 0.1% BSA and 0.05 mg/ml ascorbic acid. MCF-7 cells were harvested with trypsin/EDTA (Gibco, New York, NY.), washed twice with IMEM containing 10% FCS, re-suspended in media containing the appropriate treatment and added to the top well (20,000 cells/well). The apparatus was incubated in a humidified incubator at 37°C in 5% CO₂ / 95% air for 18 hours, after which the cells that had traversed the membrane and spread on the lower surface of the filter were stained with Diff-Quik (American Scientific Products, McGaw Pk., IL) and quantified electronically with the Zeiss IBIS 2000 Image Analysis System using a Kontron Aiag Processor interfaced with a Zeiss Axiophot Microscope equipped with an automated stage. This system analyzes 32 independent fields for each filter. Cells are identified on the basis of nuclear staining and a count of cells per field is generated. The number of cells used ensures that the cells on the lower face of the filter are not overlapping each other to a significant degree.

Invasion assays were conducted in a similar way, except that the filters were coated with 0.375 mg of Matrigel per filter (kindly provided by Dr. Hynda Kleinman, LDBA, NIDR, NIH). This layer of Matrigel was allowed to reconstitute at room temperature, resulting in the formation of a barrier through which the cells invade in order to reach the bottom side of the filter and be counted.

Correlation of PKC Isoform Levels and *in vitro* Invasive Behavior in Breast Cancer Cell Lines.

The combined approach of RNA and protein determination of PKC isoform levels was initially proposed in this project because it was not clear at the time of the application that anti-PKC

antibodies of sufficient quality and specificity would be available. The PKC proteins themselves and not the mRNAs that code for them are the functional unit their biology. Indeed, though in many cases steady state mRNA levels can be useful in determining the activity of a particular synthetic pathway, they are at least 3 steps away from describing the levels of the proteins that they code for. For this reason, when the project was initiated and it became clear that good antibodies were available, the RNA component of the study was de-emphasized. Had antibodies not been available, or of unacceptable quality, the RNA data would have been a good surrogate for protein levels in this system due to the close relationship between their levels in this system. However, due to the good results being obtained by western blot analysis those data were used in the subsequent analysis that is presented below.

Invasiveness (*in vitro*) and behavior of the cells in the nude mouse.

Initially 15 cell lines were studied in the Boyden chamber to determine their *in vitro* invasiveness to allow us to establish a suitable panel of cells for the analysis proposed, with a range of invasive potential. These cell lines were: A1N4, BT549, HS578t, MCF-7, MCF-7 ADR, MCF-10A, MDA-MB-231, MDA-MB-435, MDA-MB-435s, MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, T47D and ZR75-1. It was decided subsequently to drop the MCF-10A and the A1N4 cells from the analysis because neither cell line is in fact a tumor cell line; both being immortal mammary cell lines. BT549, MDA-MB-435s and MDA-MB-436 were also dropped due to lack of experience with these cell lines in the nude mouse. Invasion data for the remaining breast cancer cell lines in the panel, along with a description of their behavior when grown as tumors in the nude mouse are presented below. Of these, three cell lines are estrogen receptor positive (MCF-7, T47D and ZR-75-1), and the remainder are estrogen receptor negative.

Cell line	Invasiveness	Behavior in the nude mouse
Hs578t	+++++	Highly locally invasive possibly metastatic
MCF-7	++	Forms primary tumors only – no metastasis
MCF-7 ADR	+++	Forms primary tumors only – no metastasis
MDA-MB-231	+++++	Highly locally invasive possibly metastatic
MDA-MB-435	+++++	Highly locally invasive and forms metastatic deposits
MDA-MB-468	+	Slow growing primary tumors only
MDA-MB-453	+	Slow growing primary tumors only
SKBR3	+	Slow growing primary tumors only
T47D	++	Forms primary tumors only – no metastasis
ZR-75-1	+	Forms primary tumors only – no metastasis

As can be seen from this table – *in vitro* invasiveness correlates extremely well with the level of aggressive behavior that is exhibited by a particular cell line in the nude mouse. The next part of the study was to try and correlate this behavior with the level of particular PKC isoforms expressed by these breast cancer cells.

PKC Isoform Expression

Lysates of the cell lines were prepared as detailed above and subjected to western blot analysis. The expression of the various isoforms was quite varied across the different cell lines and quite

different patterns of expression were seen. For example some cell lines expressed considerable amounts of PKC alpha (Fig 1), whereas others expressed very little. Other isoforms, on the other hand, were expressed at moderate levels by all of the cell lines (Fig 2) and other isoforms were not expressed in this panel of cells at all. It became clear during these experiments, that to get data of the highest quality it was necessary to use cells lysates prepared whilst the cells were still growing – ie before confluence (as described above), as the expression of some of the isoforms appeared to be regulated somewhat by cell density. We also found that different sub-lines of the same cell line expressed different levels of the various PKC isoforms which lead us to be particularly careful the same lineage of cells was used for all assays. Hence the preparation of frozen stocks of the cells used in this study described above. The table that follows this section summarizes the expression of PKC isoforms found in the panel of breast cancer cells that were tested.

Although by the nature of the assays the values obtained do not necessarily have a linear relationship to each other, it is still useful to apply a graphical analysis to the data. Presented below (graphs 1-8) are graphical analyses of the invasion and western blot data. It is clear from this analysis that there is a relationship between the level of some of the PKC isoforms and the invasive potential of the cells and yet for others there is no such relationship.

Fig 1.

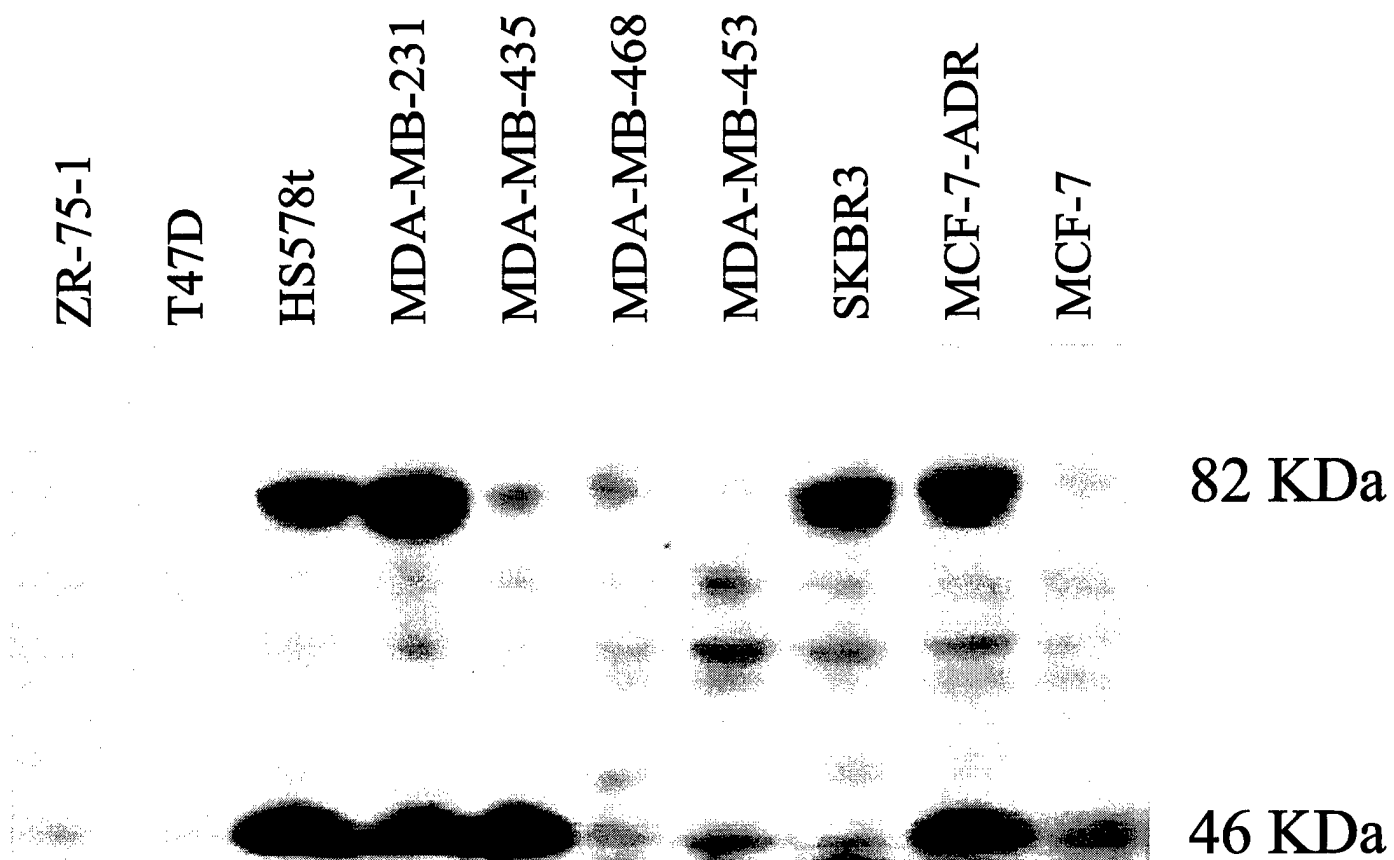
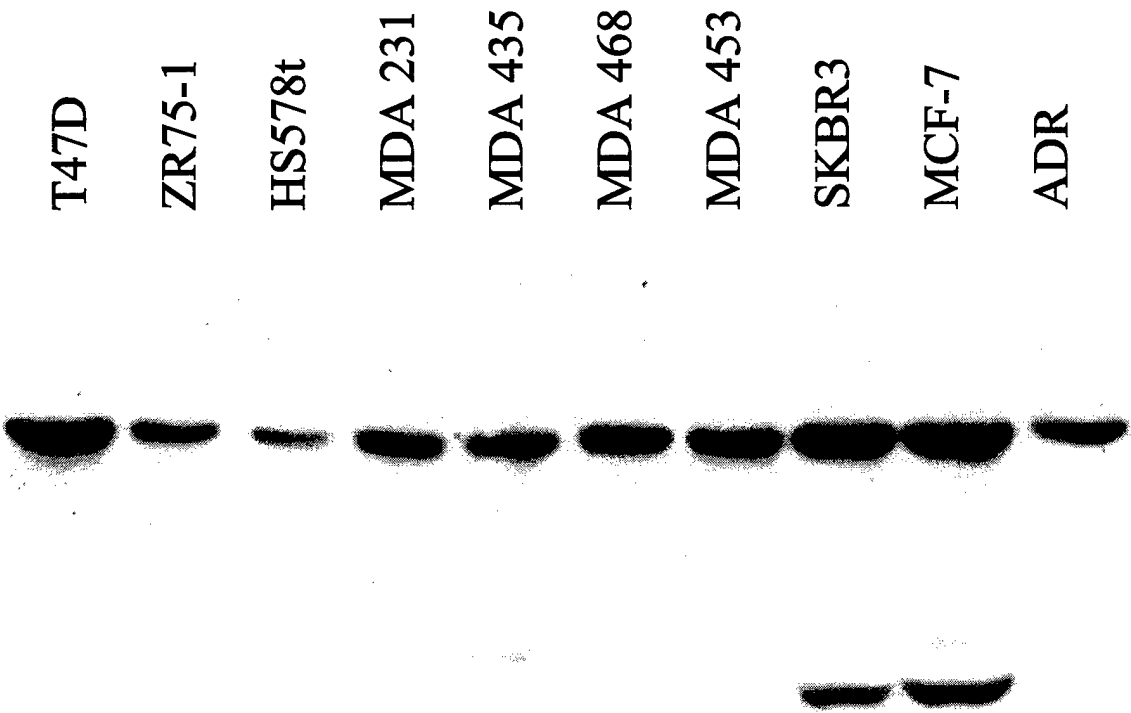


Fig 2



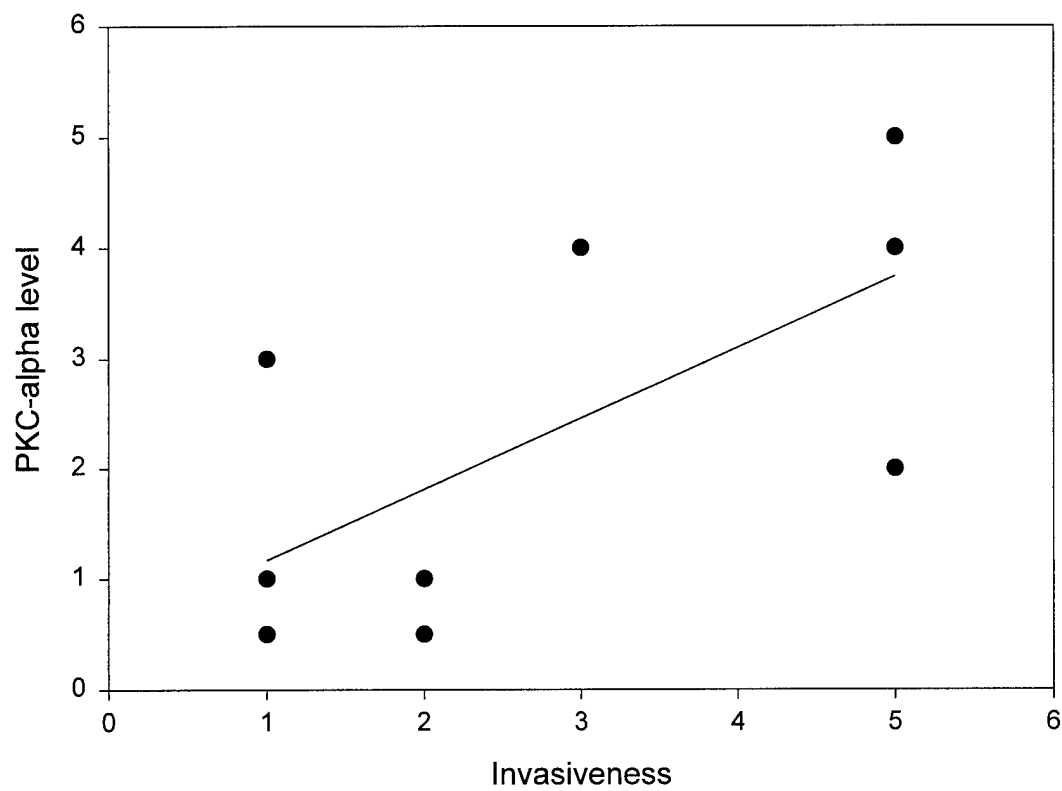
PKC Levels in Human Breast Cancer Cell Lines:

Cell Line	alpha	beta	Gamma	Delta	epsilon	eta	theta	zeta	iota	mu
Hs578t	++++	-	+	+/-	++	-	++	+++	+	-
MCF-7	+	-	+	++++	+++++	++++	+	++	+++	-
MCF-7 ADR	++++	-	+	+/-	++	+	++	+++	++	-
MDA-MB-231	+++++	-	+	+	++++	+	+	+++	+	-
MDA-MB-435	++	-	+	+++	+++++	-	+/-	+	++	-
MDA-MB-468	+	-	+/-	+/-	++	+++	++	+	++	-
MDA-MB-453	+	-	+	+++	+	+++	+	+	++	-
SKBR3	+++	-	+/-	++++	++++	++++	++	++	+++	-
T47D	+/-	-	+	++	+	++++	+	-	+++	-
ZR-75-1	+/-	-	+/-	-	++	+++	+/-	+++	++	-

The PKC isoform levels in the panel of human breast cancer cells analyzed is represented using a 1 – 5 plus scale rather than a numerical value, because it is in the nature of the western blot assay that, without very rigorous controls and the ability to run pure protein standards, it is very difficult to obtain results in terms of mass of protein. We did not have access to pure protein standards and so to represent the values as a number is a little misleading. One can, however, clearly determine by this method that some samples contain more of the protein than others do. Thus, the 5 plus scale is used to indicate these differences.

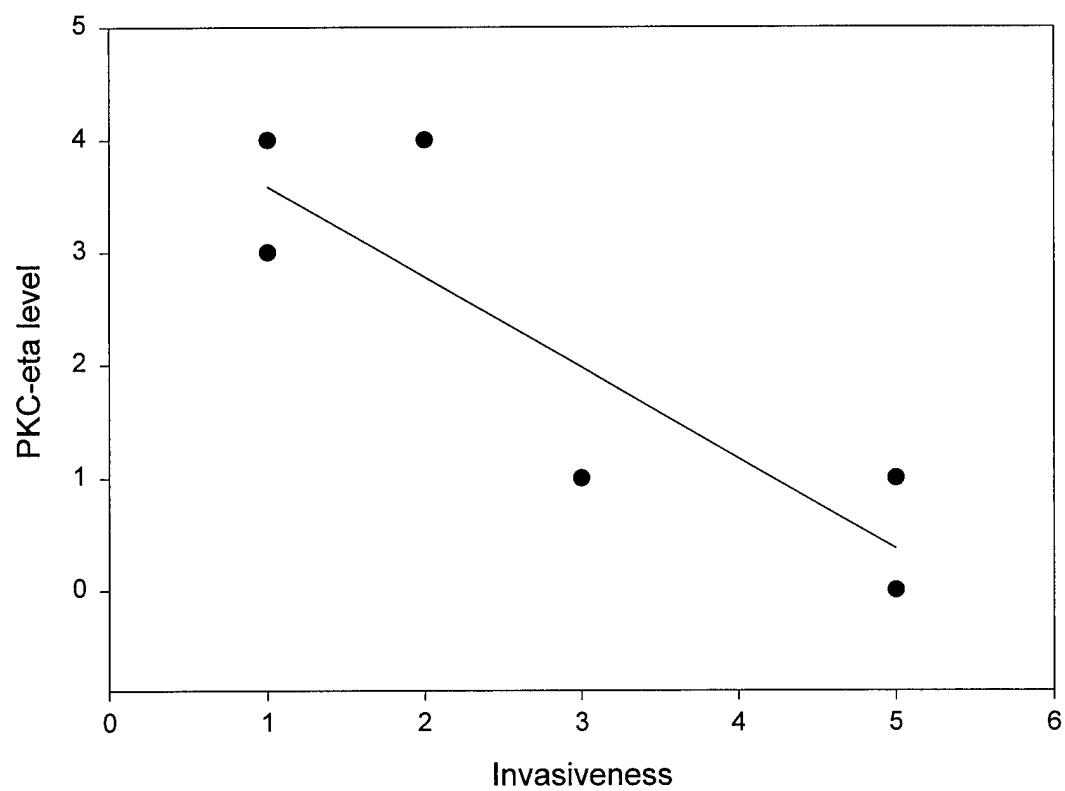
Graph 1

Correlation Between PKC-alpha Expression and Invasiveness



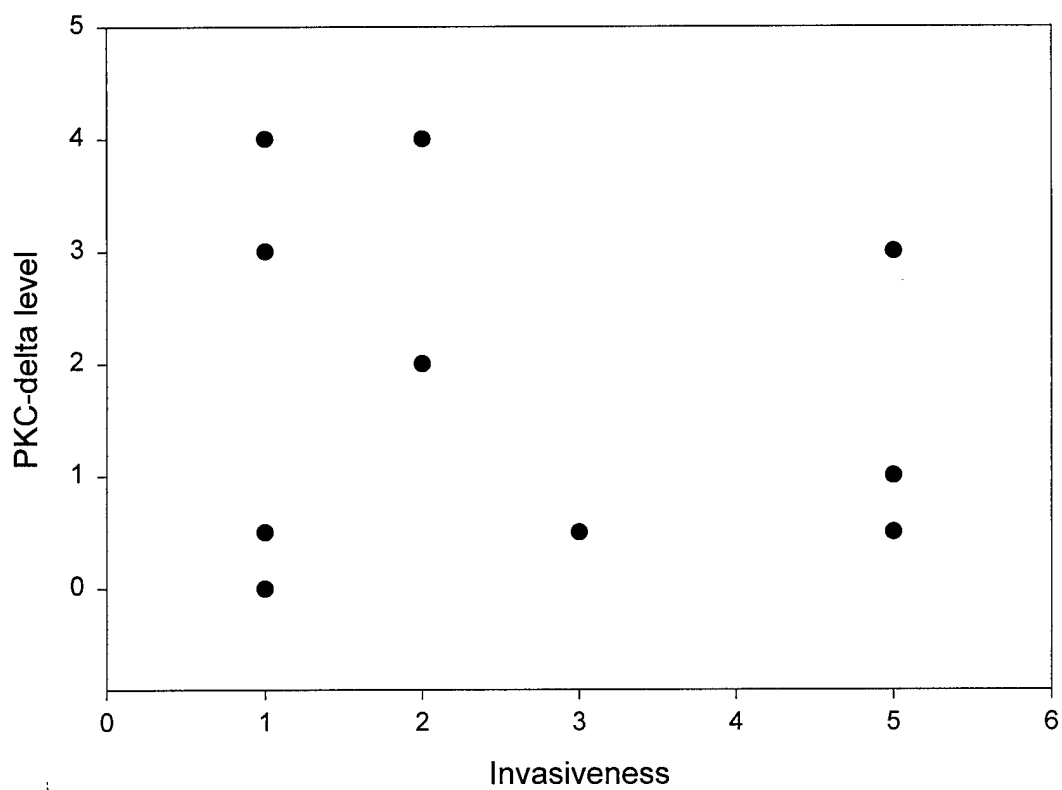
Graph 2

Inverse Correlation Between PKC-eta Levels and Invasiveness



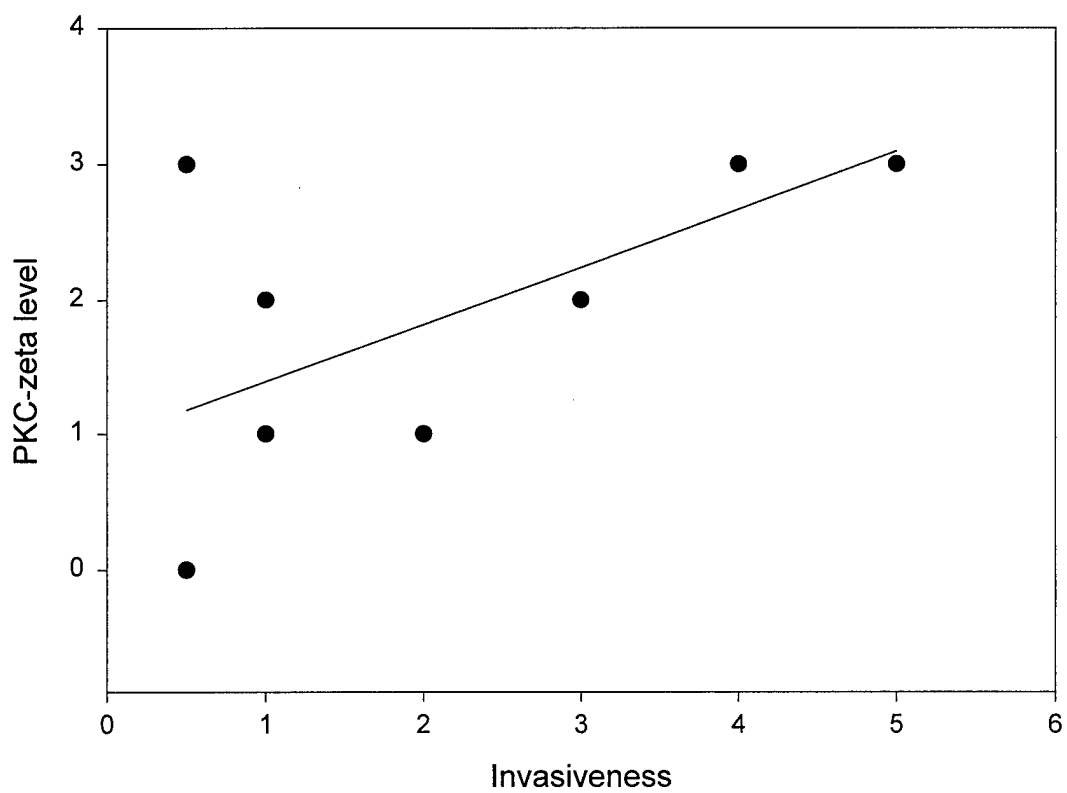
Graph 3

No Correlation Between PKC-delta Expression and Invasiveness



Graph 4

Correlation Between PKC-zeta Levels and Invasiveness



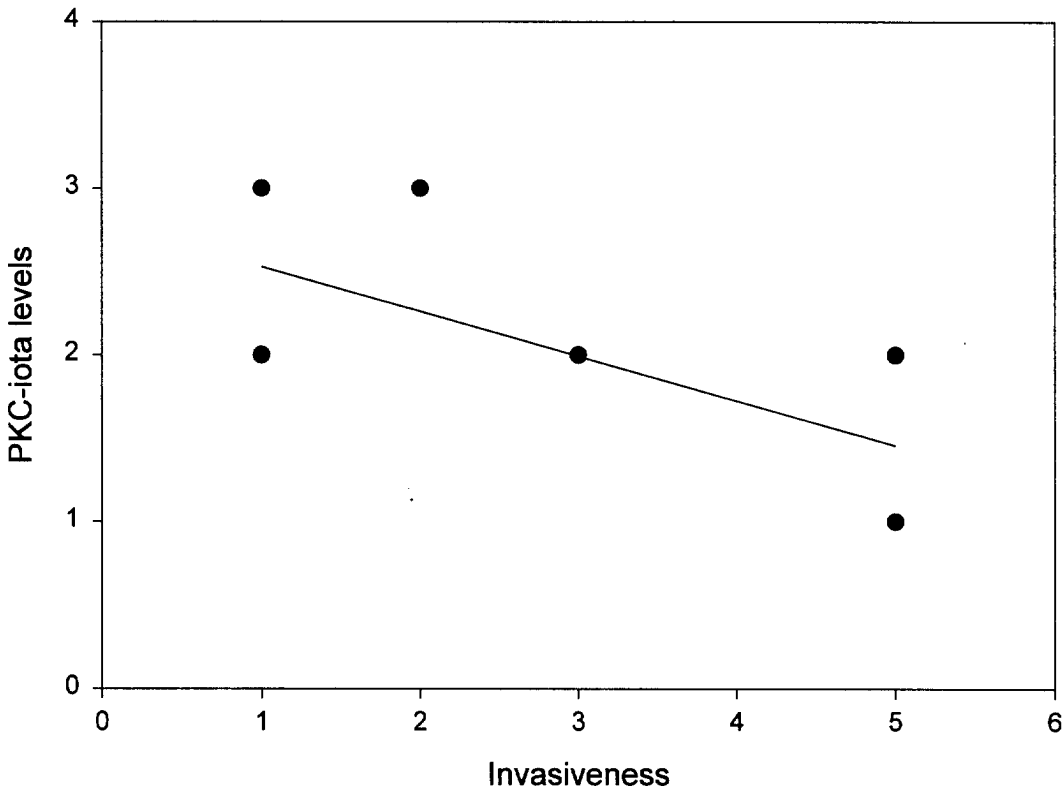
Graph 5

No Correlation Between PKC-epsilon Levels and Invasiveness



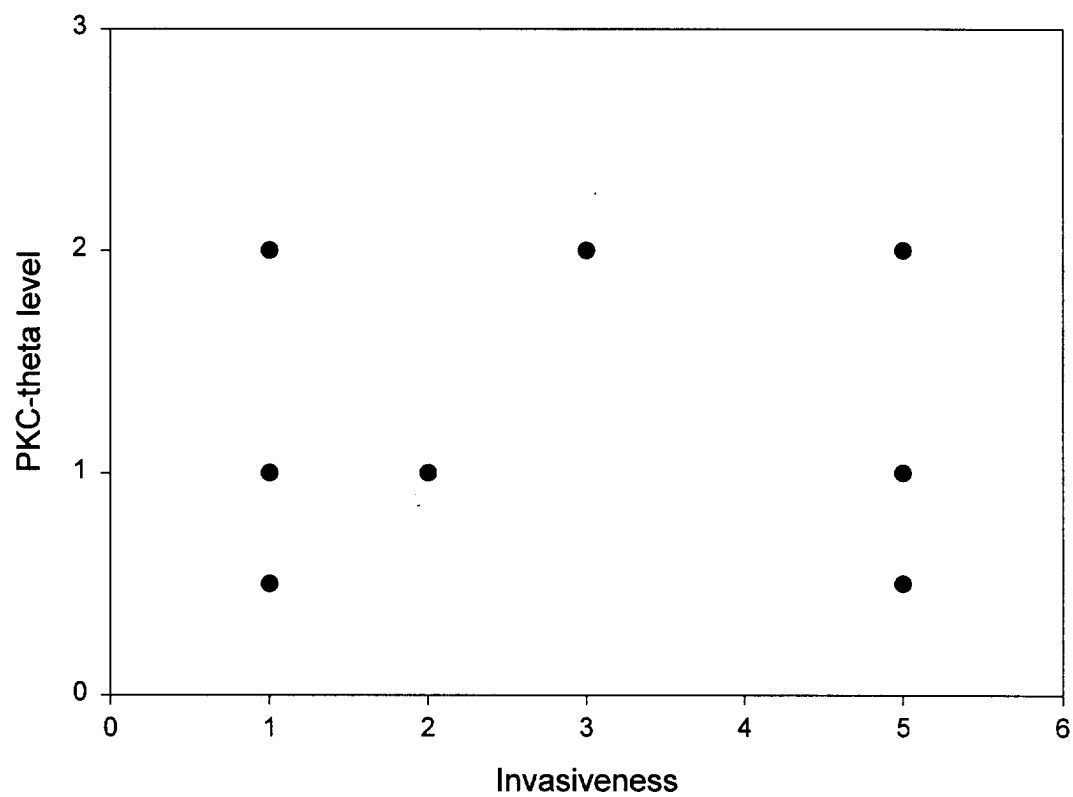
Graph 6

Weak Inverse Correlation Between PKC-iota Levels and Invasiveness



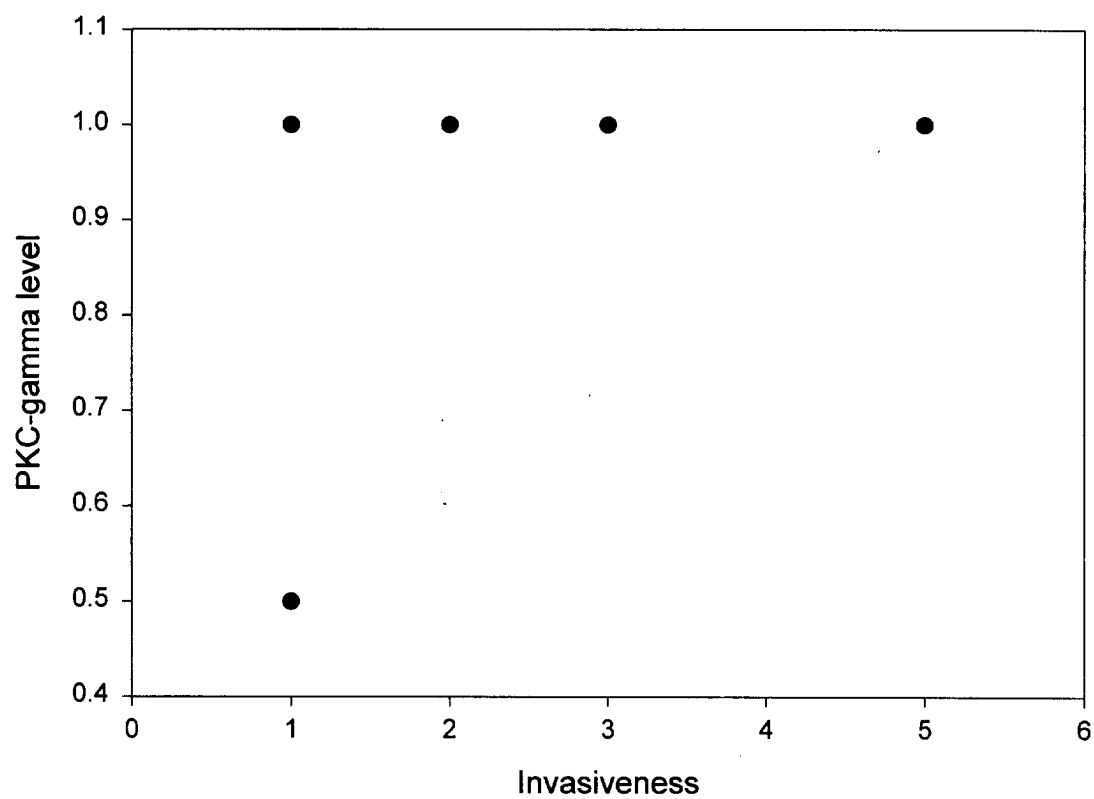
Graph 7

No Correlation Between PKC-theta Levels and Invasiveness



Graph 8

No Correlation Between PKC-gamma Levels and Invasiveness



As can be seen, there is a clear relationships between the level of PKC alpha and invasiveness, PKC zeta and invasiveness and an inverse correlation between the level of PKC eta and invasiveness.

In the original application to the USAMRDC we had proposed a series of experiments that we would conduct when we had determined which isoforms of PKC were associated with a more invasive and metastatic phenotype. However, whilst proposal was under review and whilst we were conducting the experiments described above, several things happened that led us to request a change in the emphasis of the proposal. The first of these was the publication of a paper in which the first of our proposed transfection experiments was conducted (3). The poorly invasive, non-metastatic breast cancer cell line MCF-7 was transfected with PKC-alpha. From our data, presented above, we would predict that this intervention would result in the cells becoming more invasive and metastatic. Gratifyingly, this was indeed the result. Although there were some flaws in this study, it nevertheless seemed to us to be an unnecessary duplication of the work of others to repeat the experiment. Particularly since to fully characterize the cell lines that we would generate would require the use of a significant number of nude mice.

Similarly, we had originally proposed to examine the effect of the drug Bryostatin-1 on the levels of PKC protein and activity in human breast cancer cell lines grown as tumors in nude mice. Bryostatin-1 was only in the earliest phases of clinical trial when we proposed our experiments. However, by the time the first Aim of the proposal had been completed, the drug was about to enter a later stage trial within the Developmental Therapeutics (DT) program in our own clinic. This presented the exciting prospect of being able to achieve many of the goals of our proposed animal experiments in the much more relevant setting of a clinical trial in humans. Furthermore an additional anti-PKC therapeutic agent was soon to be tested in the DT clinic providing us with the opportunity to compare the effects of two agents with distinctly different mechanisms of action. We therefore, with the approval of the Institutional Review Board (IRB) set in place plans to obtain samples from the patients engaged in these trials and redirected the scope of our proposal to take advantage of this exciting opportunity.

Modulation of PKC Isoform Levels and Activity by Therapy with Bryostatin-1

Background:

Bryostatin-1 is a member of a family of macrocyclic lactones isolated from the marine bryozoan *Bugula neritina* (See attached structure). The compound binds to the same portion of PKC the molecule that the PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) interacts with and can compete for TPA binding and, therefore, can interact with all c- and n-type isoforms. The EC50s (50% Effective Concentration) that are reported for the antagonism of various PKC mediated effects are quite varied and probably reflect different affinities for the different PKC isoforms that may be mediating these effects. The mechanism of Bryostatin-1's inhibition of PKC activity is still somewhat unclear. It probably occurs in two main ways that may occur simultaneously depending on the system examined. It is thought that interaction of Bryostatin-1 with the PKC protein results in a very rapid, transient activation of the activity of the protein that in turn results in the rapid destruction and removal of the protein from the cell. In this way, the PKC protein is ablated from the cell resulting in inhibition of PKC mediated signaling. Alternatively, the high-affinity binding of Bryostatin-1 to the PKC molecule blocks the binding

of a co-factor that is necessary for PKC activity, and down-stream signaling is thereby blocked. In most cases it is likely that both mechanisms are important.

We had a tremendous amount of pre-clinical experience with Bryostatin-1, having conducted a significant number of *in vitro* experiments with Bryostatin-1, some of which were used as preliminary data for the original proposal. However, before the clinical trials started we extended these studies to guide us as to sample selection, preparation and analysis in the clinical-correlative studies. Some of this work is described below:

Pre-clinical Studies with Bryostatin-1

Ablation of PKC alpha from cells treated with Bryostatin-1

Initial studies were conducted using the MCF-7 ADR human breast cancer cell line that was used above. This cell line was chosen because it is one of the more aggressive cell lines and has good expression of PKC-alpha. To examine if Bryostatin-1 resulted in the rapid ablation of PKC alpha, MCF-7 ADR cells were treated with increasing concentrations of Bryostatin-1 in IMEM with 10% FCS for various periods of time. The drug was dissolved in ethanol, the final concentration being 0.1% and 0.1% ethanol was added to control cells. At the end of the treatment periods the cells were lysed as described above and 20 ug aliquots of the samples run on 10% SDS PAGE gels and transferred to membranes. The membranes were probed with the anti-PKC alpha antibody as described above. From the data shown in Fig 3 it can be seen that treatment of MCF-7 ADR cells for 1 hour with 1 nM or greater concentrations of Bryostatin-1 results in the complete loss of PKC alpha from the cells.

It has been known for some time that some of the Matrix Metallo Proteases (MMPs) are PKC regulated in some systems. We therefore decided to determine if the transcription and synthesis of MMP-9 was altered by treatment with Bryostatin-1. We had previously shown that MMP-9 mRNA was TPA inducible in MCF-7 cells and so we set out to determine if this induction could be antagonized by treatment with Bryostatin-1. MCF-7 cells were treated with increasing concentrations of TPA and then TPA in combination with Bryostatin-1. Conditioned medium was collected from the cells and total RNA was prepared from the cell as described above. The RNA was fractionated and the level of MMP-9 mRNA was determined by northern blot analysis. The conditioned medium from the cells was analyzed for the presence of MMP-9 by gelatin zymography (see below for description of the method). As can be seen in Fig 4, treatment with TPA produced a marked increase in the level of both MMP-9 mRNA and in secreted MMP-9 protein. Determination of MMP-9 levels by zymography is as reliable a measure as mRNA levels in determining the effects of Bryostatin-1 on the system.

We also decided to examine the activity of one of the down-stream signaling pathways on which PKC is believed to exert its effects, the Mitogen Activated Protein Kinase (MAPK) pathway. Measuring the activity of this signaling pathway should give us an indication of the level of activity of PKC in the cells which may well not be the same as the amount of protein found within them. Cells were treated for various periods with several concentrations of Bryostatin-1 and cell lysates were made as before. Equal amounts of protein 50 ug were loaded and run on duplicate 12% SDS PAGE gels and transferred to nitrocellulose membranes. After blocking in 5% NFDm for 4 hours at room temperature the membranes were exposed to either an antibody that recognizes total MAPK protein or an antibody that recognizes only the active

phosphorylated form of the MAPK protein. After extensive washing the membranes were exposed to an anti-rabbit HRP conjugated antibody and areas of immunoreaction detected with the use of ECL reagent and x-ray film. Representative data are shown in Fig 5 and it is clear that although the level of total MAPK is not significantly altered by the treatment with Bryostatin-1 (1nM in this experiment), there is a marked decrease in the level of MAPK phosphorylation.

From these data and other reports in the literature we decided that in the initial clinical trial of continuous infusion of Bryostatin-1 we would examine changes in the levels of PKC isoforms, alterations in MMP-9 levels and alterations in the degree of MAPK phosphorylation as markers of Bryostatin-1 action. The nature of the initial clinical trial meant that it would be difficult to obtain tumor tissues for analysis, though this would be a goal of subsequent trials (see below). For this reason it was decided to look at MMP-levels in the plasma from the patients at various periods before and during therapy – we already had considerable experience with such assays from previous studies (4). For the PKC assays it was decided to use the tissue that the drug first comes in contact with – IE the blood – and so PKC levels in the Peripheral Blood Lymphocytes (PBLs) were measured. The same samples could also be used for the MAPK activity assays. In addition, should the tumor be accessible, biopsies would be taken for direct assays of the various end-points.

Fig 3

- MCF-7 cells treated with the indicated concentration of Bryostatin for 1 hour
- Whole cell lysate prepared
- Analyze equal amounts of protein for PKC- α expression by western.

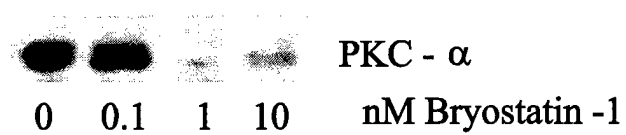


Fig 4

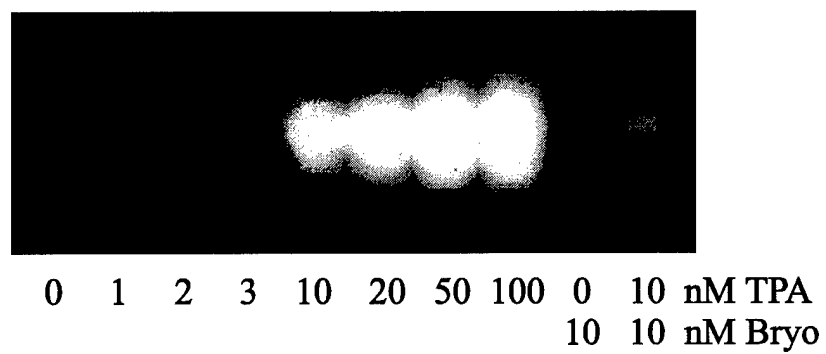
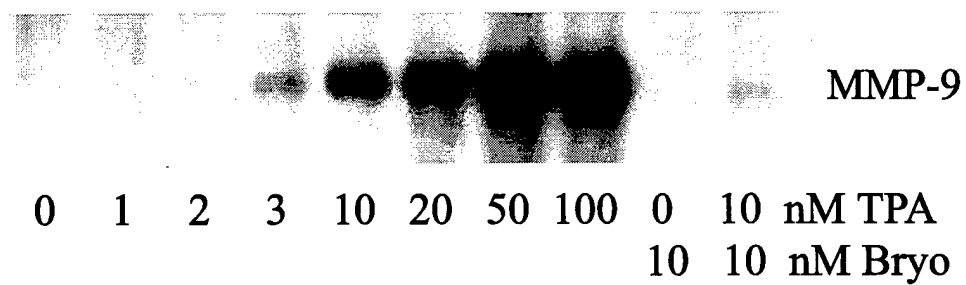
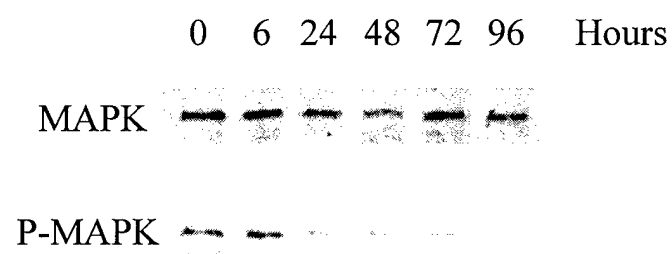
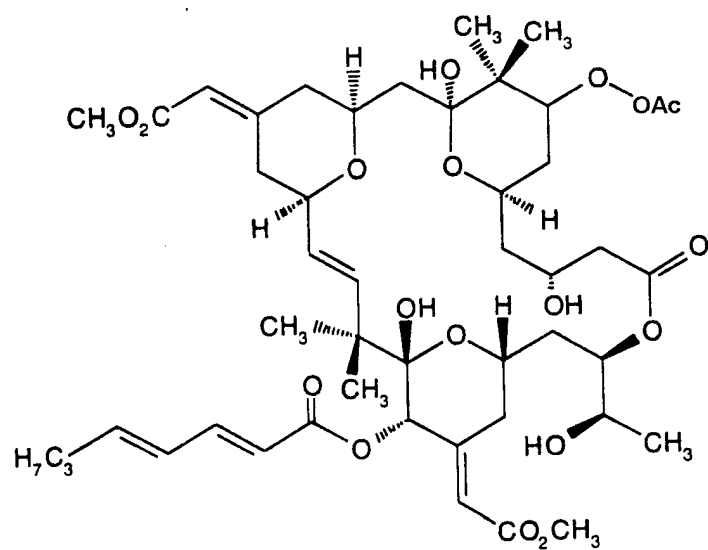


Fig 5



Bryostatin-1



Bryostatin-1 clinical trial.

An initial phase 1 trial of Bryostatin-1 was conducted within the DT clinic in the Lombardi Cancer Center. The PI of this study was Dr John Marshall and the analysis of the samples from the trial was conducted as a collaboration with him. All protocols were approved by the Georgetown IRB in accordance with normal procedures. Attached are the eligibility criteria for this study which are fairly standard for a phase 1 study. The median age of patients included was 59, ranging from 43 – 79 and all patients had received and failed on extensive prior therapy. Bryostatin-1 was administered in an increasing dose and length of infusion schedule as outlined until dose limiting toxicity (myalgia, fatigue, pain and malaise) halted dose escalation.

Blood samples were drawn from patients before the commencement of therapy and at various times during and after the infusion of the drug (see below). Blood for the preparation of plasma was prepared using tubes containing EDTA as the anticoagulant, spun at room temperature and then aliquoted and frozen at –80C until analyzed. PBLs were prepared using standard methods. Briefly, patient blood collected in EDTA containing tubes was diluted 1:1 with an equal volume of PBS and layered onto Ficoll Paque (Pharmacia, Picataway, NJ) and then spun at room temperature for 40 minutes at 400 x G. After this spin, the plasma was aspirated and discarded and the PBL layer removed, diluted with PBS and spun again to remove the platelets. The PBL pellet was then lysed with 1 ml of boiling lysis buffer (10mM Tris pH 7.5, 1% SDS, and to inhibit any phosphatase action: 5mM sodium pyrophosphate, 100uM Sodium vanadate, and 50mM sodium fluoride). After boiling for 10 minutes the lysate was cooled and a sample removed for protein assay. The remaining sample was diluted 1:1 with 2 X SDS PAGE loading buffer, aliquoted and stored at –80C until analyzed.

Sample analysis

MMP-9 assays

MMP-9 levels were determined by zymography using methods we have described before (4). Briefly, patient plasma samples were diluted in 1 x zymogram loading buffer and run, without boiling on 10 % SDS PAGE gels containing 0.1% gelatin. The gels were subsequently washed twice for 30 minutes in Tris buffered saline containing 2% Triton X-100, and then incubated overnight at 37oC in 50 mM Tris pH 7, 5mM calcium chloride, 1% Triton X100, to allow gelatin degradation. Areas of digestion were then visualized by staining with Coomassie blue R250 in 10% acetic acid/20% methanol, followed by de-staining in 10% acetic acid/20% methanol. The gels were then dried and the areas of clearing measured by image analysis. The data from these assays are shown below.

As can be seen from these data there was very considerable variation in the level of MMP-9 in the patient plasma. MMP-2 levels which can also be seen on the same zymograms did not alter much and so act as a useful control to show that indeed equal amounts of sample were loaded on each track. Thus, the level of variation that is seen is not an artifact of unequal loading. We have been unable to determine any pattern that can link the level of MMP-9 seen in the patients plasma with exposure to Bryostatin-1 and conclude that the noise of the natural variation of this enzyme in human plasma is too great to allow interpretation of the data. However, we have recently developed an assay for Bryostatin-1 that is sensitive enough to allow the levels of

Bryostatin-1 in these samples to be determined and so it is possible that when these assays are conducted we may yet be able to determine an informative pattern.

PKC assays.

PKC assays were conducted essentially as described above for the cell line work. Equal amounts of PBL lysate (20ug) were fractionated on 7% SDS PAGE gels and transferred to nitrocellulose membranes. After blocking, exposure to the primary anti-PKC antibodies, areas of immunoreaction were detected using HRP conjugated secondary antibodies and ECL reagents. The level of each PKC for each time-point was then estimated by scanning densitometry.

The results of these assays are shown below. As can be seen, there is a significant level of variation in the PKC levels and it is hard at first analysis to determine any pattern to these alterations in PKC levels. We believe that, in part, this high degree of variation is due to subtle shifts in the sub-populations of PBLs that make up the protein in the lysates, since we know that the different cells contain different levels of the various isoforms. Nevertheless, despite this variation, there is a clear pattern for one of the isoforms. The levels of PKC-eta are consistently suppressed by treatment with Bryostatin-1 in most patients as can be seen in summary in Fig 6. As with the MMP-9 data, it may be that other more subtle patterns will emerge when the pharmacokinetic analysis is completed. We are in the process of exploring further the suppression of PKC-eta levels in a new clinical trial that has been developed from this initial trial (see Below).

MAPK activity assays.

The same lysates prepared for the PKC assays have been used for MAPK activity assays. It was to facilitate this sort of study that phosphatase inhibitors were added to the lysis buffer to protect these labile groups. The assays were done more or less as described for the preliminary experiments. Equal samples of lysate (50ug) were loaded onto duplicate SDS PAGE gels, run and transferred to nitrocellulose. The membranes were blocked with 5% NFDM in PBST and analyzed with the antibodies that recognize total MAPK and phospho MAPK. A representative blot is shown in Fig 7. As can be seen in this figure there is not difference in the level of total MAPK in the different time points and very interestingly, there is no alteration in the level of phospho-MAPK. This was a highly consistent finding – levels of total and phospho MAPK were essentially unchanged during therapy. This suggests that despite the alterations in PKC levels and activity being produced by the Bryostatin-1, that this had no effect on signalling through the MAPK pathway. This interesting finding is under further investigation, and we believe may represent a difference between cells of epithelial and hematologic origin.

As described above, it was the plan to obtain samples of any accessible tumor samples from patients on the trial. In the event, only one patient had a tumor site that was amenable to obtaining samples, however, we were fortunate enough to be able to obtain samples before, during and after the completion of the Bryostatin-1 infusion. This patient was on dose level 3 and so was receiving 24ug/sqm over 96 hours. Tumor samples were obtained and immediately flash frozen to -196C. They were then stored at -80C until they were analyzed. Tumor lysates were prepared by powdering the tumor samples under liquid nitrogen. The tumor powders were then suspended in boiling lysis buffer (10mM Tris pH 7.5, 1% SDS, and to inhibit any phosphatase action: 5mM sodium pyrophosphate, 100uM Sodium vanadate, and 50mM sodium

fluoride) and boiled for 10 minutes. A sample of each lysate was removed for protein determination and the remainder diluted 1:1 with 2 X SDS PAGE loading buffer. The lysates were aliquoted and frozen at -80C until analyzed.

PKC levels were determined in these lysates in exactly the same way and for the PBL lysates and MAPK activity assays were also conducted as for the PBL lysates. Some of the data obtained with these data are shown in Fig 8. As can be seen, PKC alpha levels were very low in the initial, pre-treatment sample and then are markedly increased in the sample taken during therapy and further increased after the end of therapy. There is a similar increase in the level of PKC eta during therapy, though after the drug infusion, PKC eta levels fall slightly. Total MAPK levels increase across the three samples whereas phospho-MAPK is only during therapy. These data are quite hard to interpret and may in part be a function of the limitations of the sample gathering. The tumor that was samples was growing very rapidly throughout the study and was quite necrotic in places. It is possible that the quality of the samples was rather different and that this has skewed the results. We propose to do further western blot analysis using markers that may allow us to control for this possibility. The rise in total MAPK throughout the study again suggests that this is a viable explanation. Alternatively, the somewhat paradoxical results that are seen may be an indication of the lack of sensitivity of this tumor to this therapy. As described above, the tumor continued to proliferate unchecked throughout the therapy and other tumor deposits in the patient were apparently as unaffected by the treatment. It may be that the paradoxical effects that were seen are symptomatic of this apparent resistance.

In summary, the data that were generated from this initial trial of Bryostatin-1 were somewhat disappointing in that the clear differences that we had hoped for were not readily evident. However, some interesting findings were generated and, as alluded to several times above, studies are continuing with the samples that should allow us to obtain a better picture of what in fact is going on. As described above, we have now developed a Bryostatin-1 assay of sufficient sensitivity to determine the concentrations of drug achieved in each patient at each time-point. Though not directly part of this project, this data should greatly facilitate the interpretation of our results and represents a significant technical achievement that other labs have not been able to duplicate. Although this is a final report, as the results from these continuing studies are presented, the support from the USAMRDC will, of course, be gratefully acknowledged.

As mentioned above, this initial trial has led to additional trials examining the efficacy and action of Bryostatin-1. In particular, we are in the process of studying the impact of continuous infusion of 8ug/msq of Bryostatin-1. In this trial we are looking at PKC levels in the PBLs of the patients, but we have also put in place a mechanism to allow us to get a better idea of what is happening in the tumors of these patients. Specifically, we will obtain needle biopsies of the tumors before and during the infusion. These samples will be embedded in paraffin and sectioned. We will then conduct a series of immunohistochemical studies to look at the levels of various PKC isoforms, markers of apoptosis and proliferation and the activity of signal transduction pathways. This study will make use of the methods that we developed for the PKC anti-sense trial described below. When this work is published we will of course gratefully acknowledge the support of the USAMRDC.

Phase 1 Trial of Bryostatin-1:

Eligibility Criteria

- | <u>Inclusion Criteria</u> | <u>Exclusion Criteria</u> |
|--|--|
| • Histologically confirmed, incurable malignancy | • Recent major surgery (within 21 d) |
| • ECOG PS 0, 1, or 2 | • Clinically active CNS disease |
| • WBC > 3000, ANC > 1500, Platelet > 100,000 | • Frequent vomiting |
| • Normal PT, PTT, Bilirubin, Creatinine +/- Cr Clearance | • Severe anorexia |
| • Anticipated survival > 8 weeks | • Recent weight loss > 10% |
| • > 18 years old | • Pregnant or lactating women |
| • Able to sign informed consent | • Serious intercurrent illness |
| | • Concurrent radiation, chemotherapy, or immunotherapy |

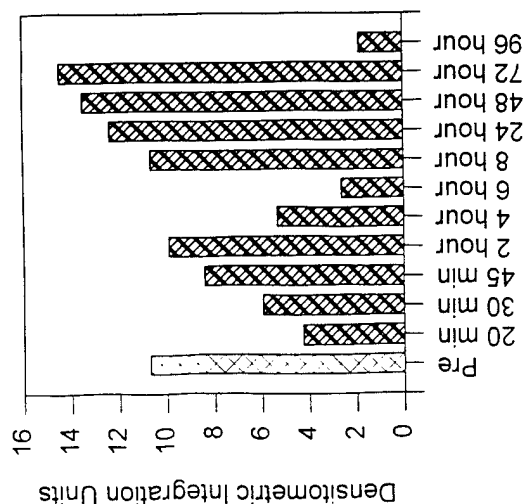
Phase 1 Trial of Bryostatin-1: Dose Levels

- Dose Level 1 8 ug/m²/day over 96 hrs
- Dose Level 2 16 ug/m²/day over 96 hrs
- Dose Level 3 24 ug/m²/day over 96 hrs
- Dose Level 4 24 ug/m²/day over 120hrs
- Dose Level 5 24 ug/m²/day over 144hrs
- Dose Level 6 24 ug/m²/day over 168hrs
- Dose Level 7 32 ug/m²/day over 168hrs

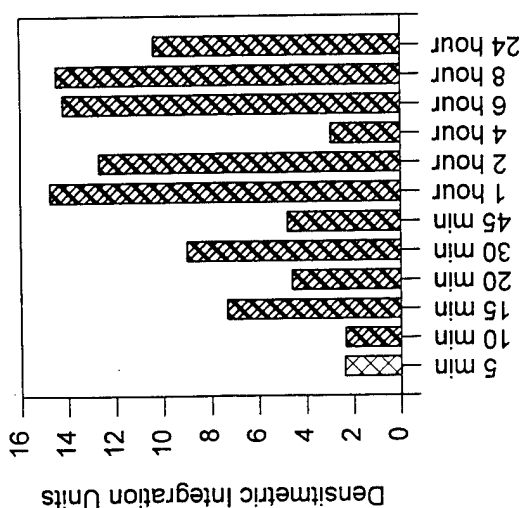
Phase 1 Bryostatin-1: Toxicity

- Grade 1-3 myalgias, fatigue, pain, malaise
- Grade 1-2 nausea, anorexia, diarrhea, constipation
- No myelotoxicity seen
- All toxicity has been reversible
- Myalgias/fatigue required 1-4 weeks to reverse

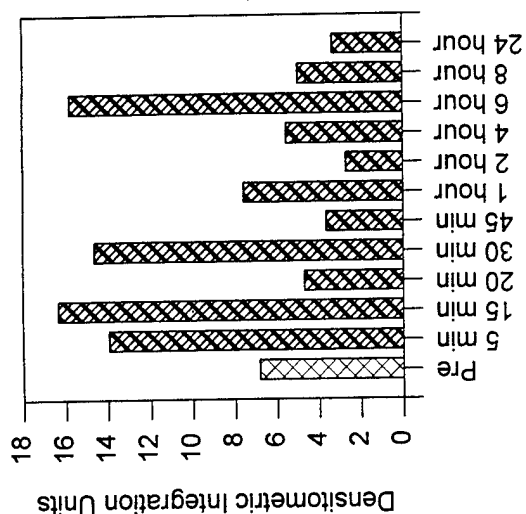
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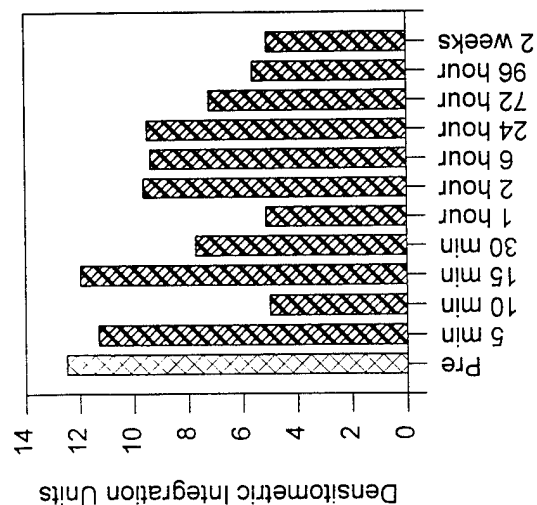
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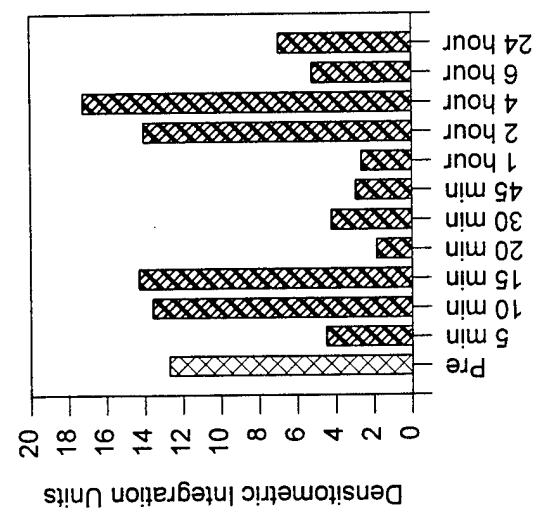
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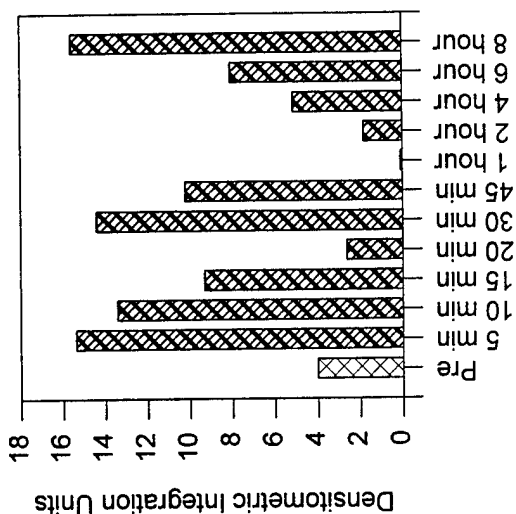
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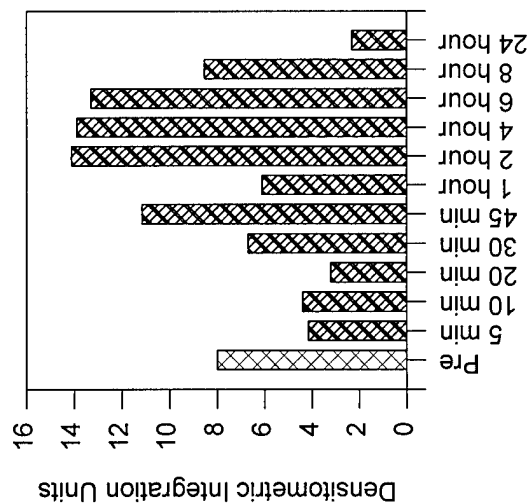
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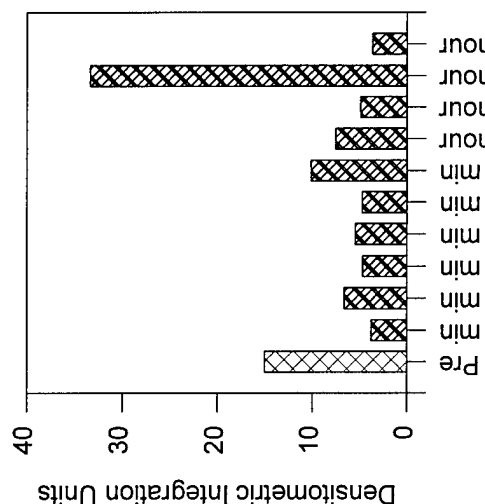
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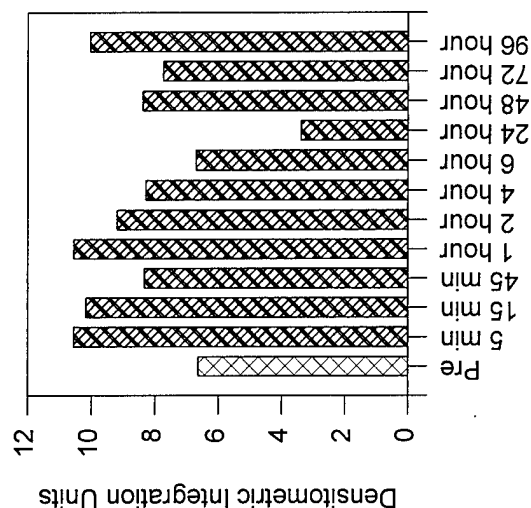
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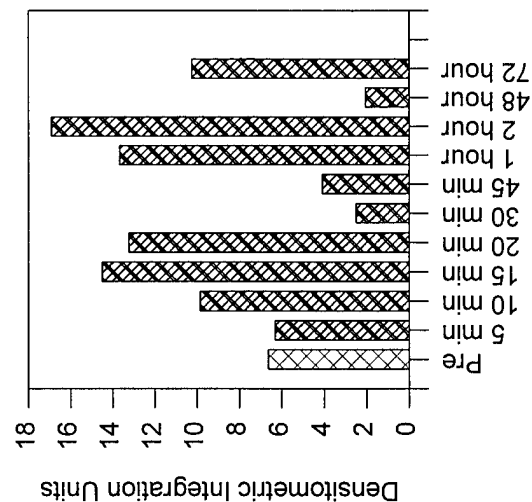
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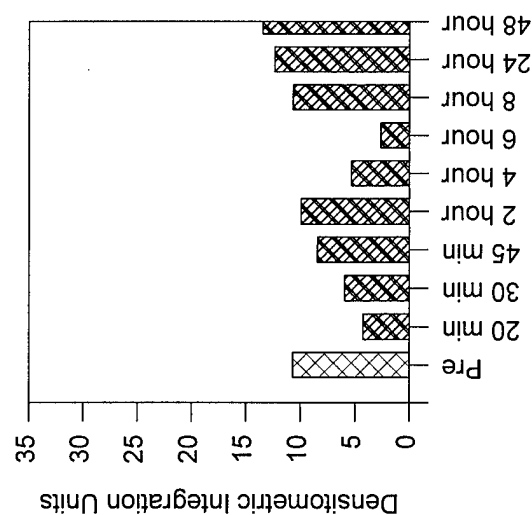
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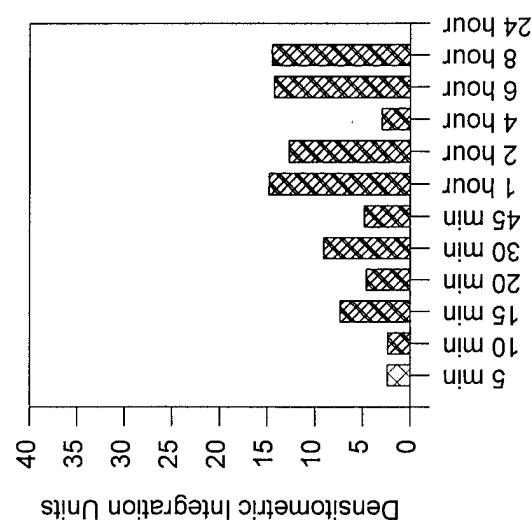
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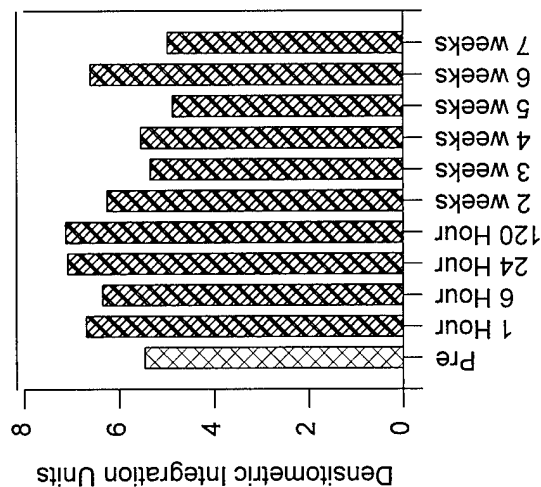
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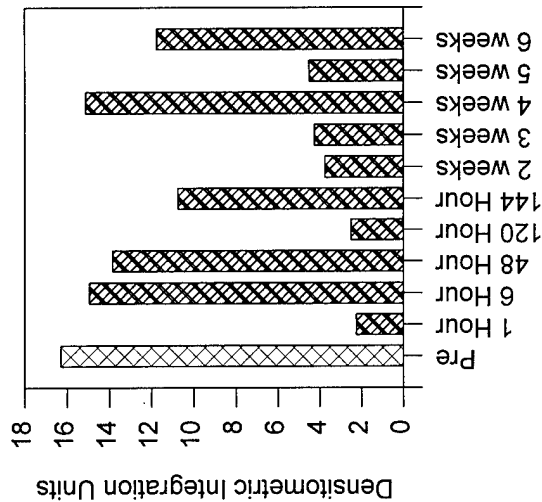
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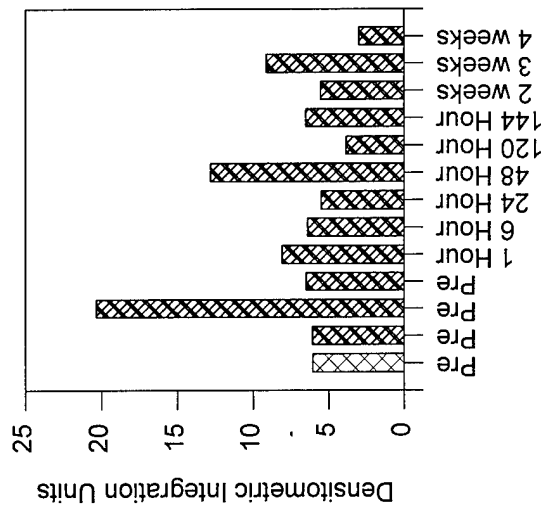
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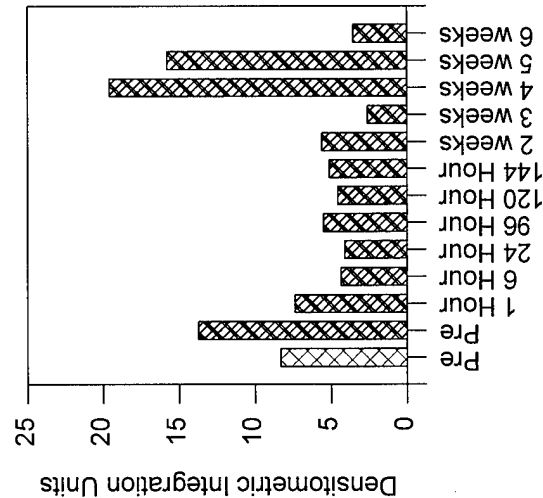
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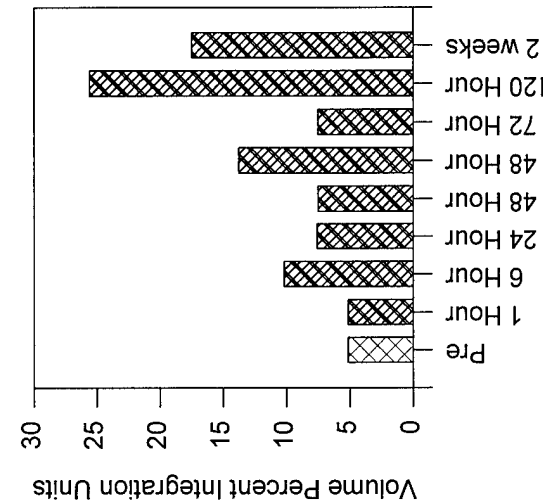
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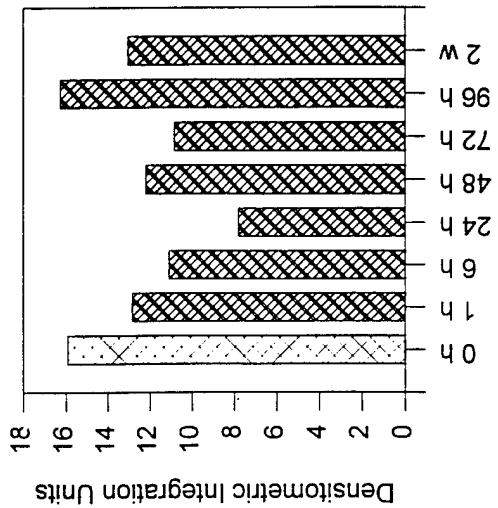
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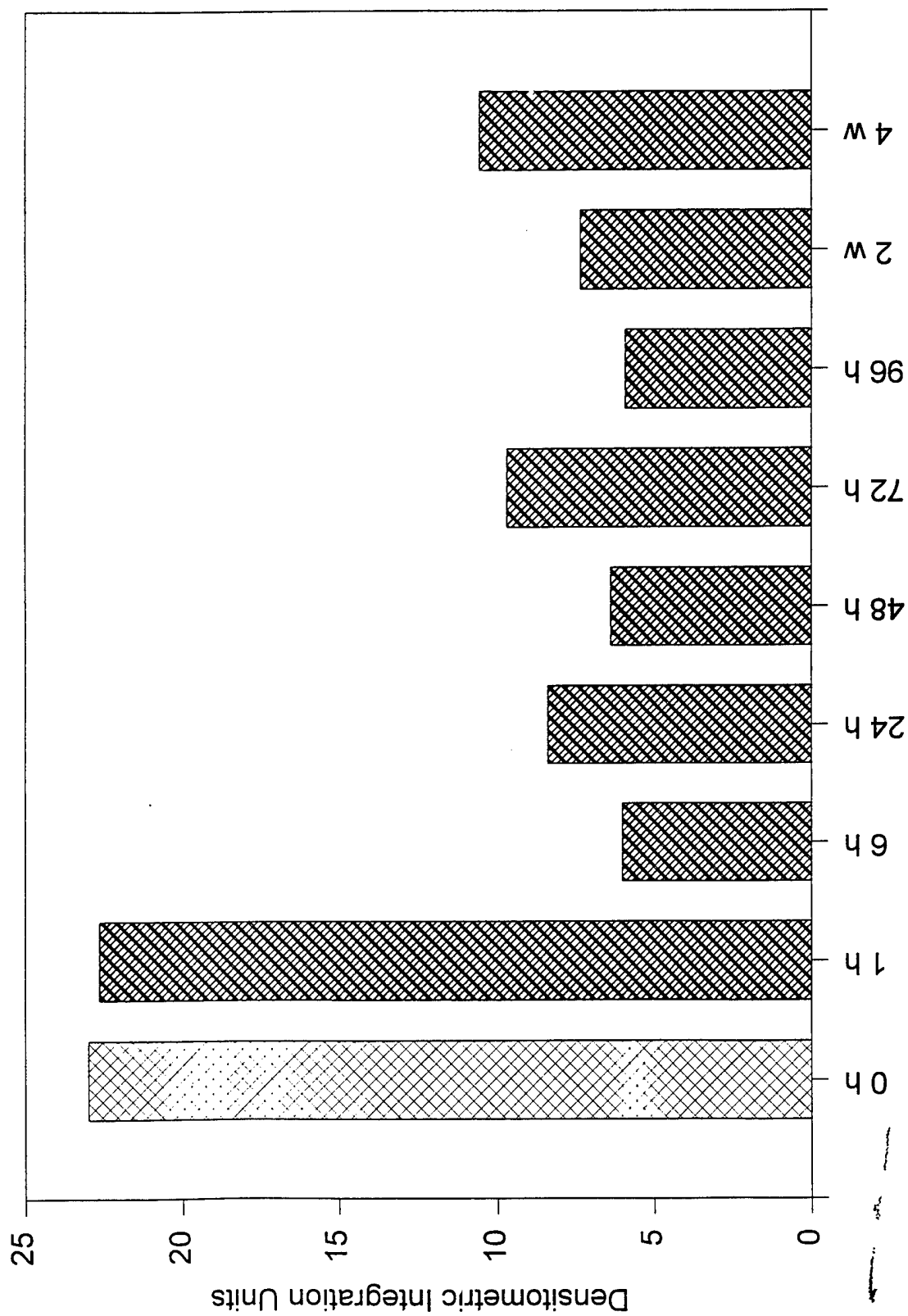
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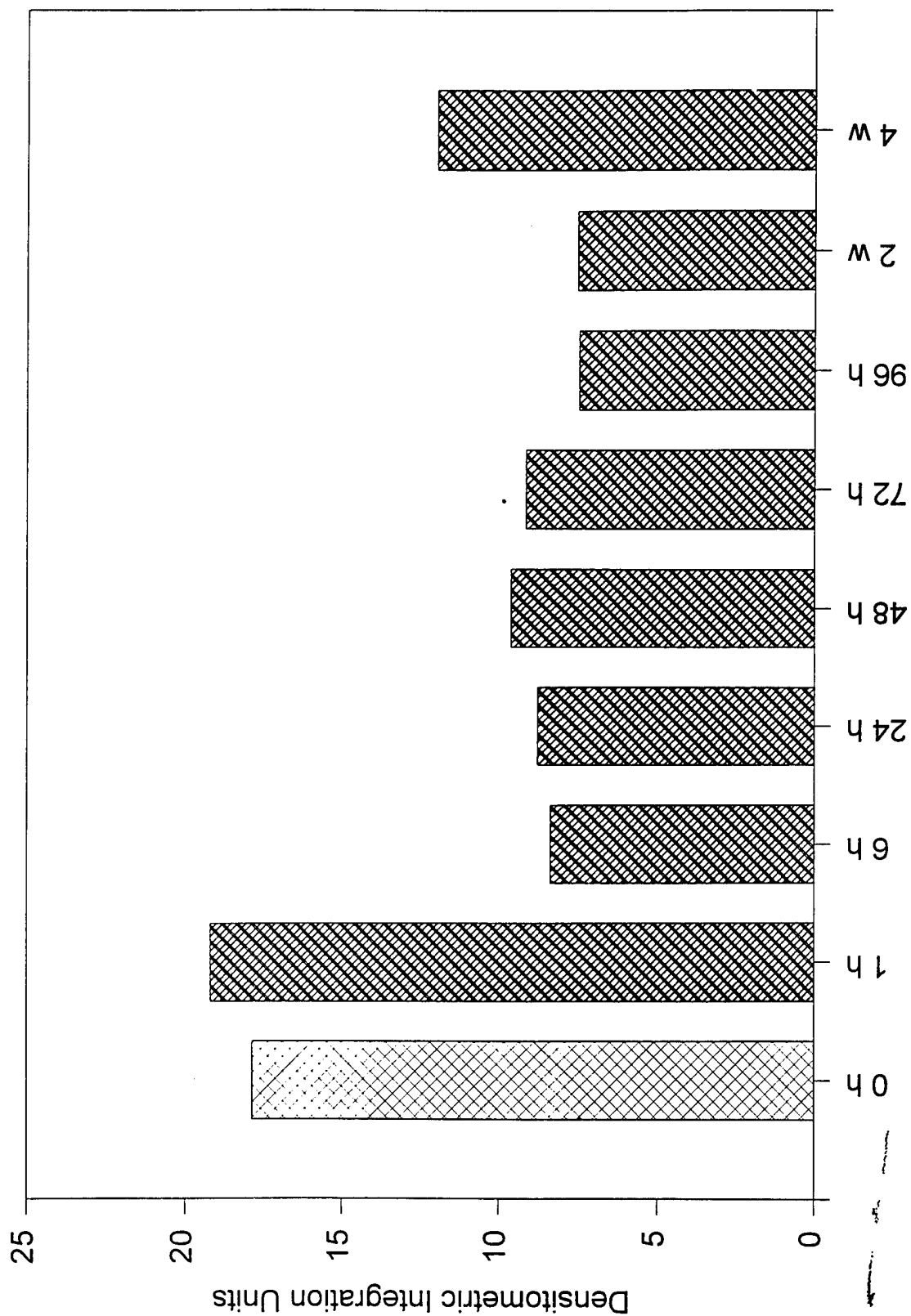
PKC-alpha



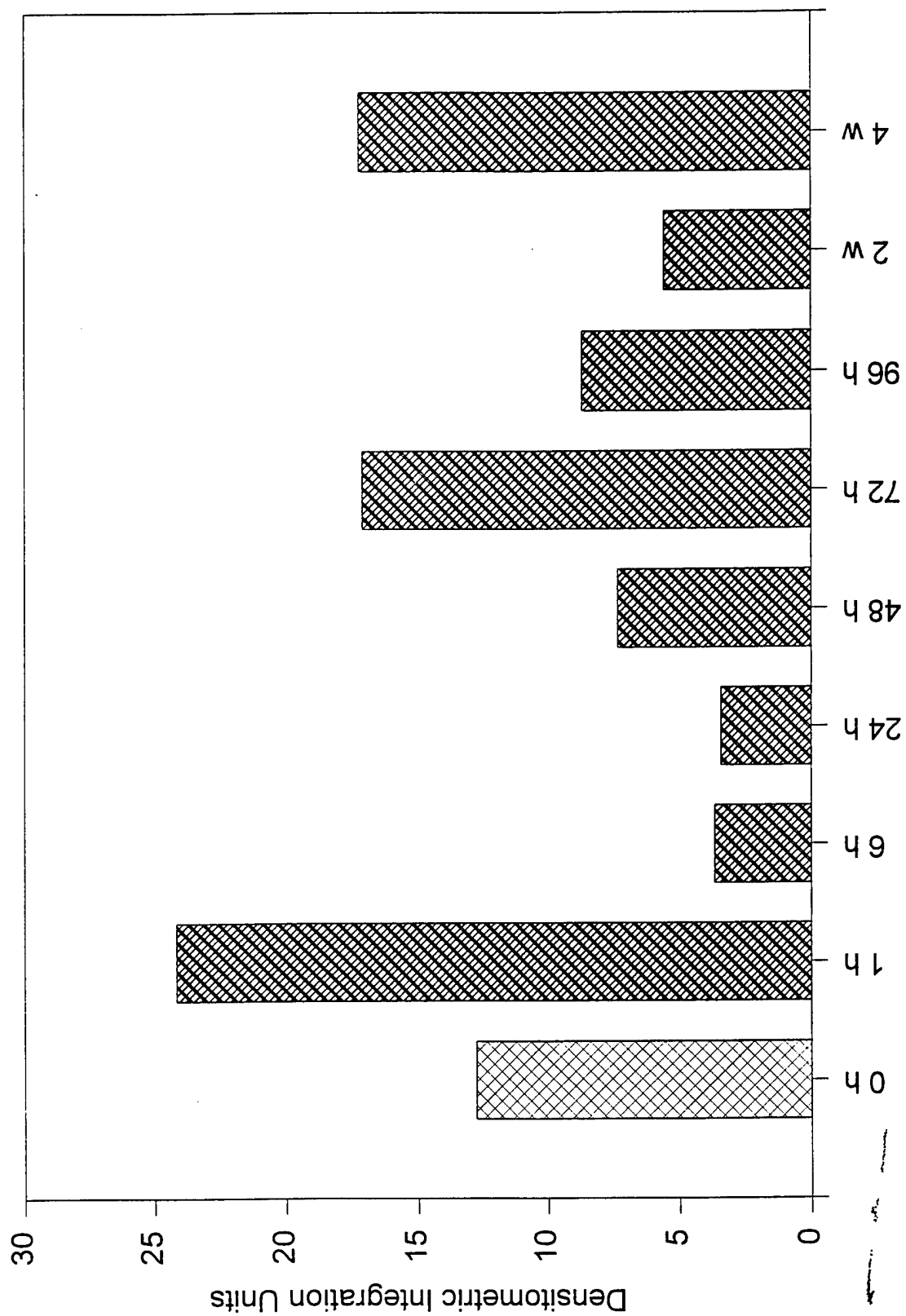
PKC-delta Levels in Patient #4 vs. Treatment with Bryostat-1



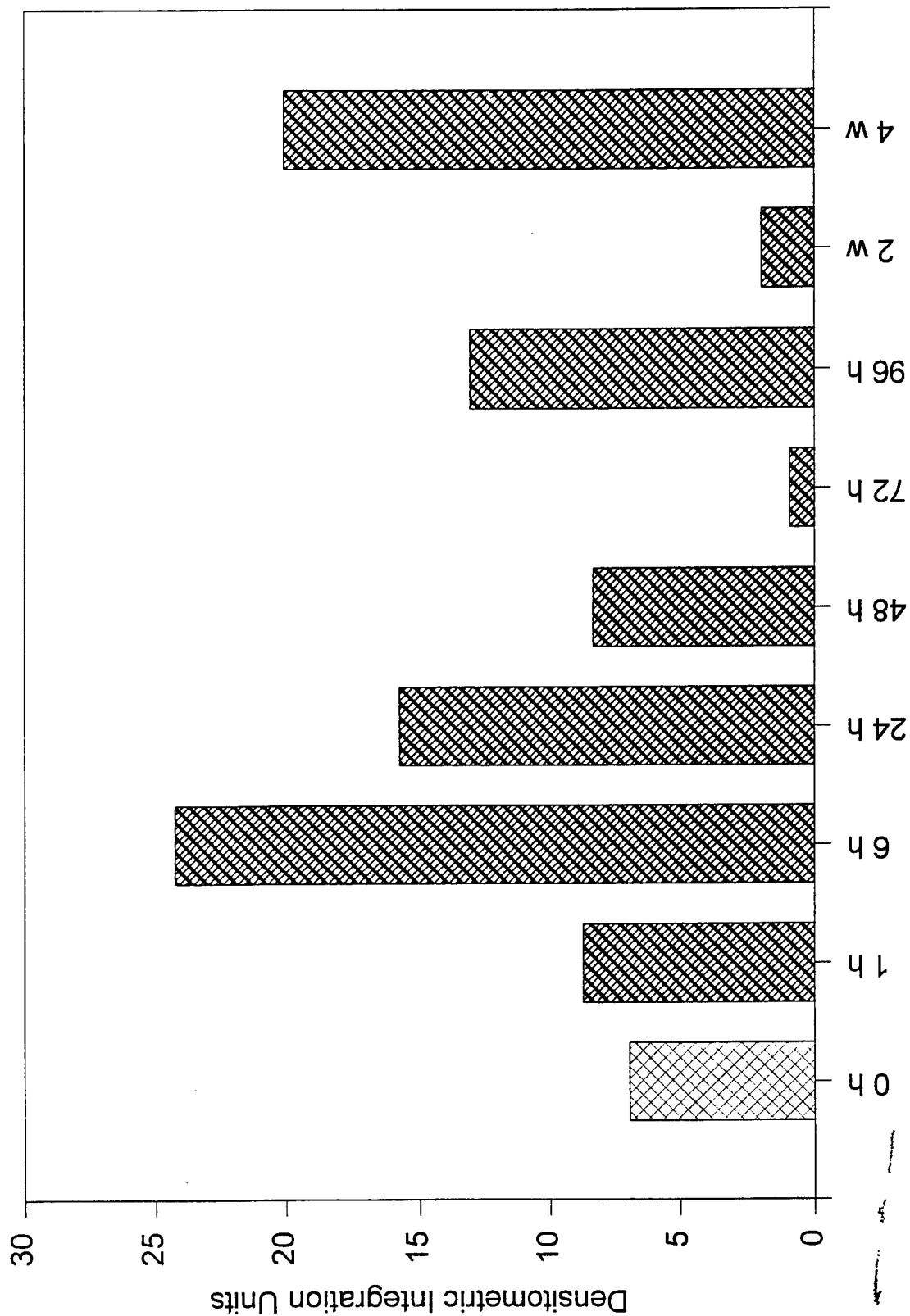
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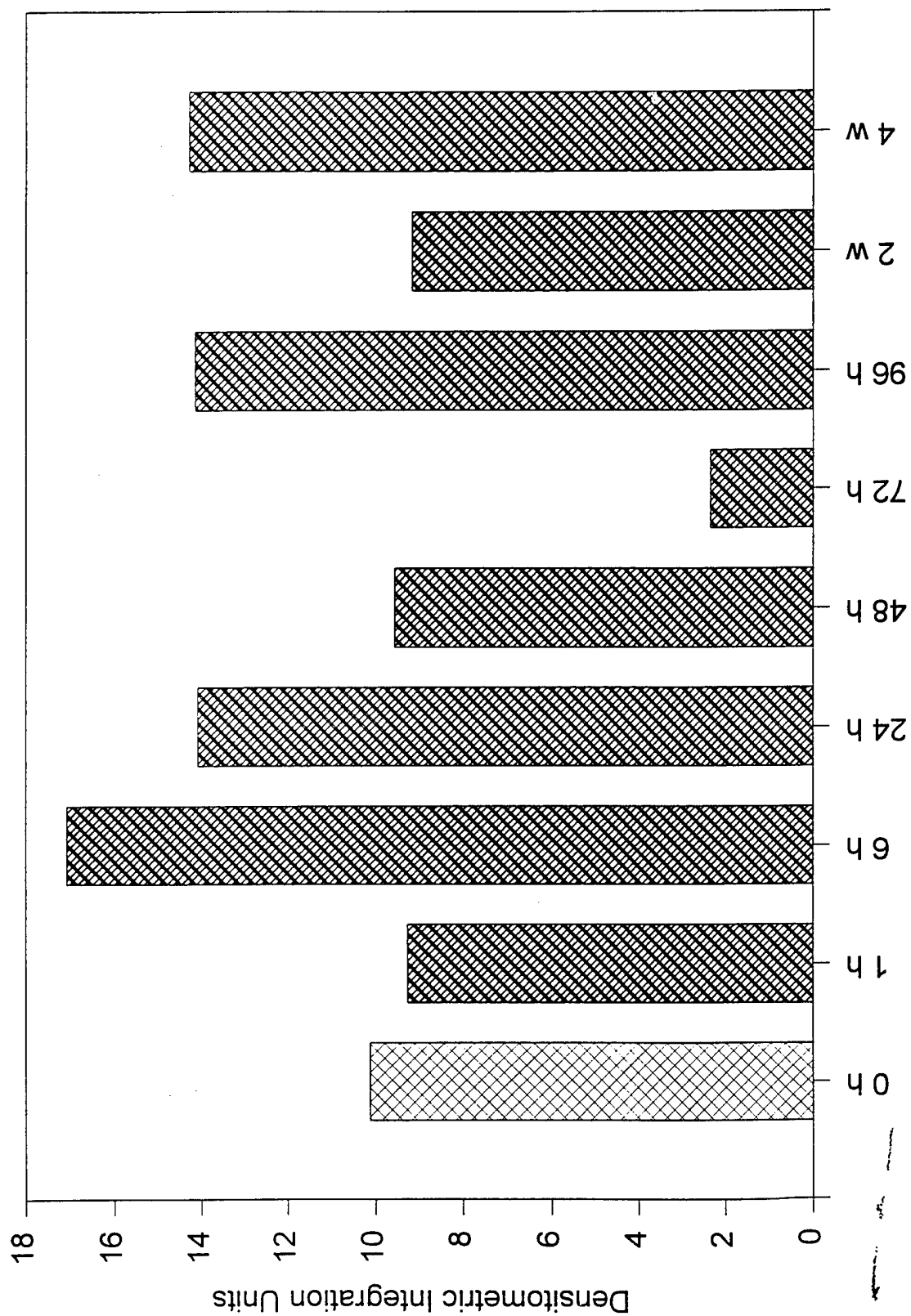
PKC-eta Levels in Patient #4 vs. Treatment with Bryostat-1



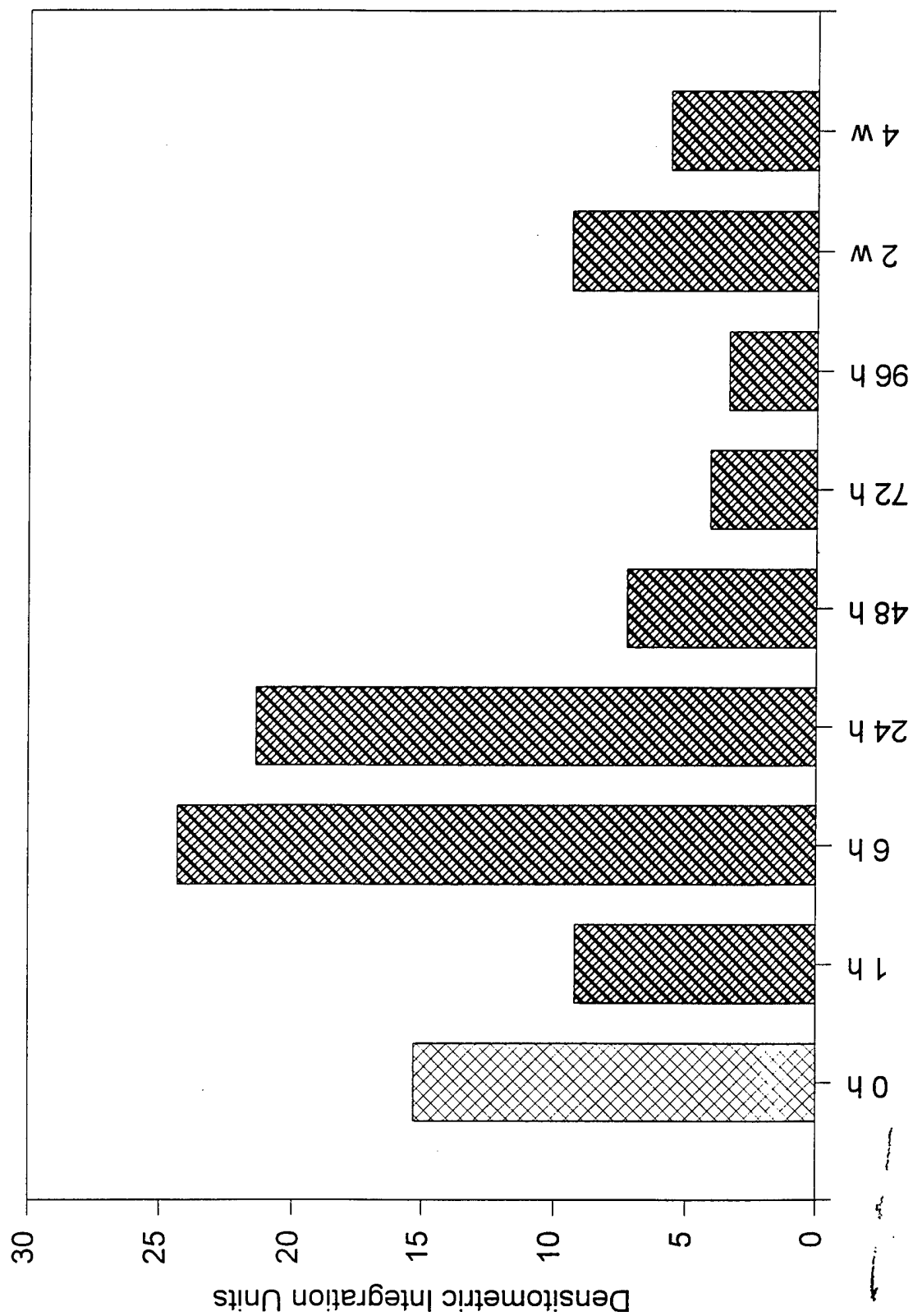
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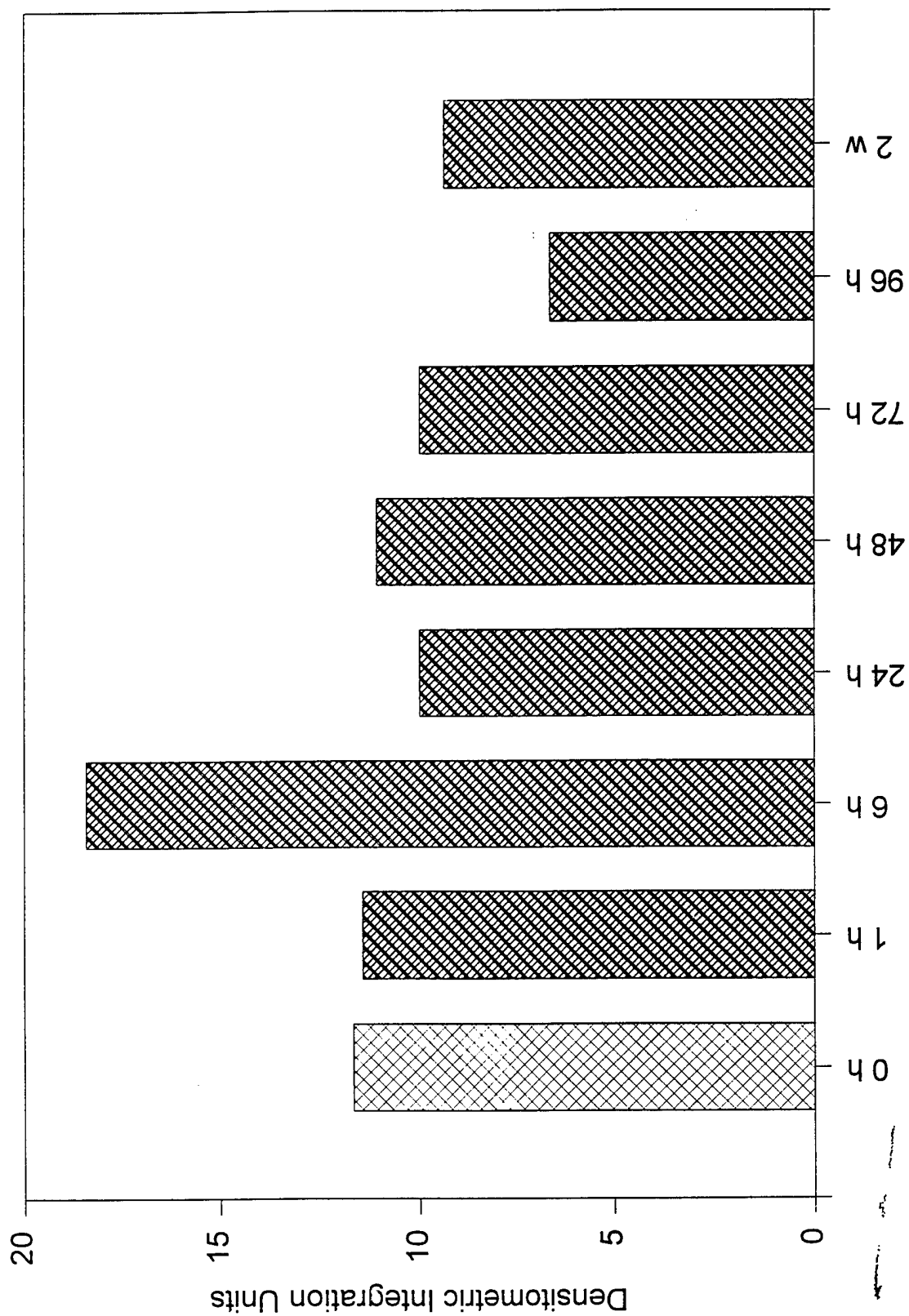
PKC-epsilon Levels in Patient #6 vs. Treatment with Bryostat-1



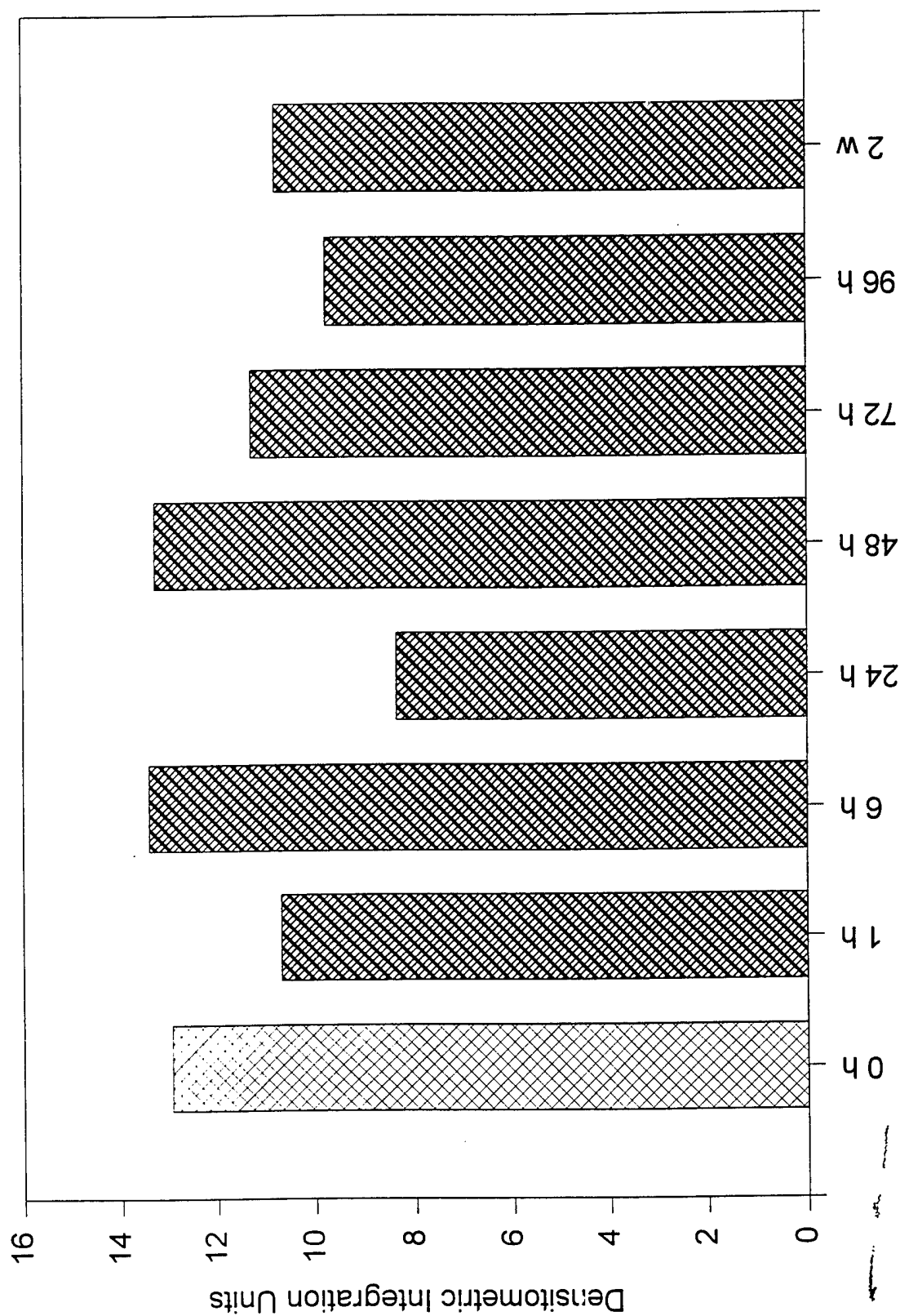
PKC-eta Levels vs. Treatment with Bryostatin-1



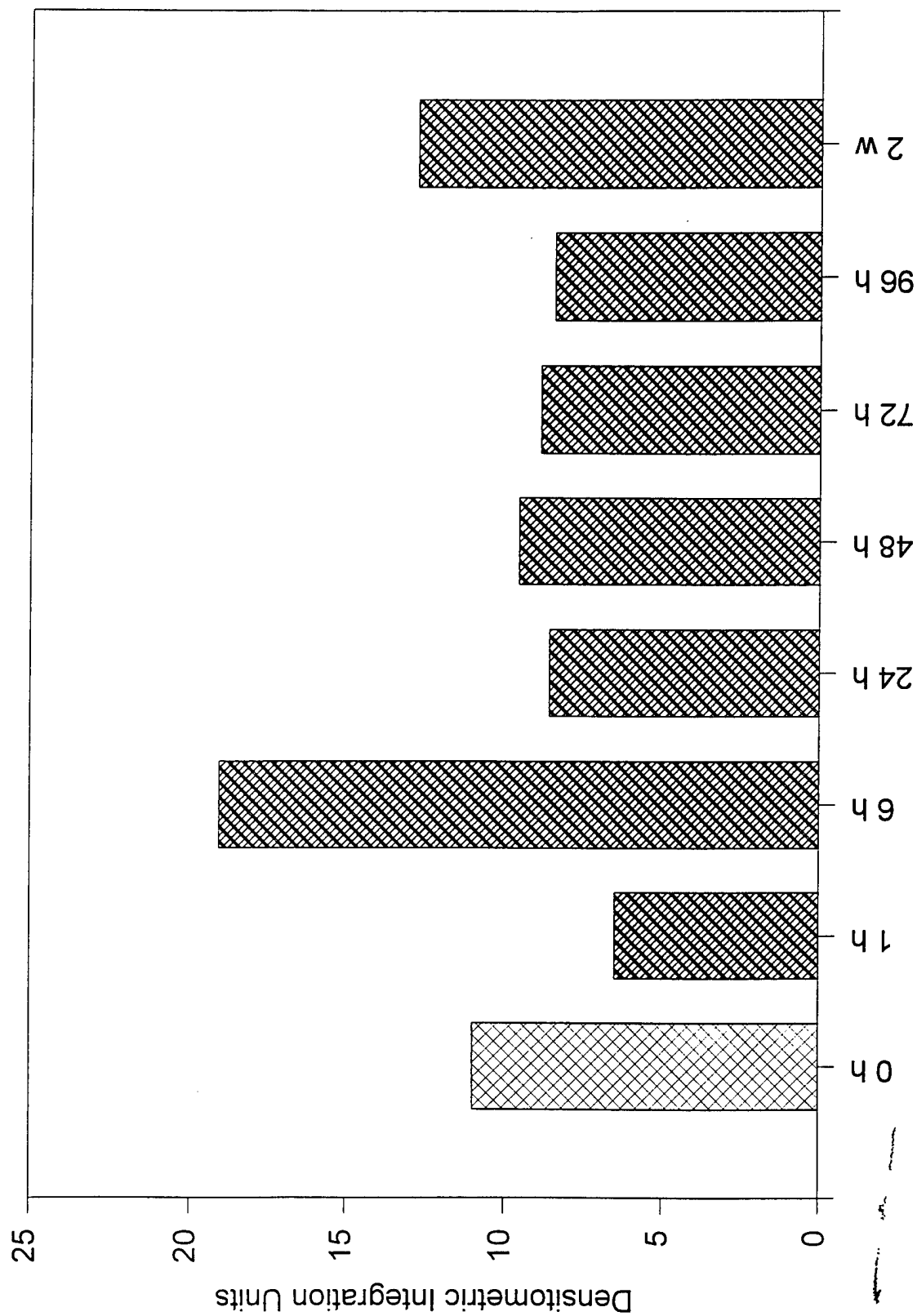
PKC-delta Levels in Patient #7 vs. Treatment with Bryostat-1



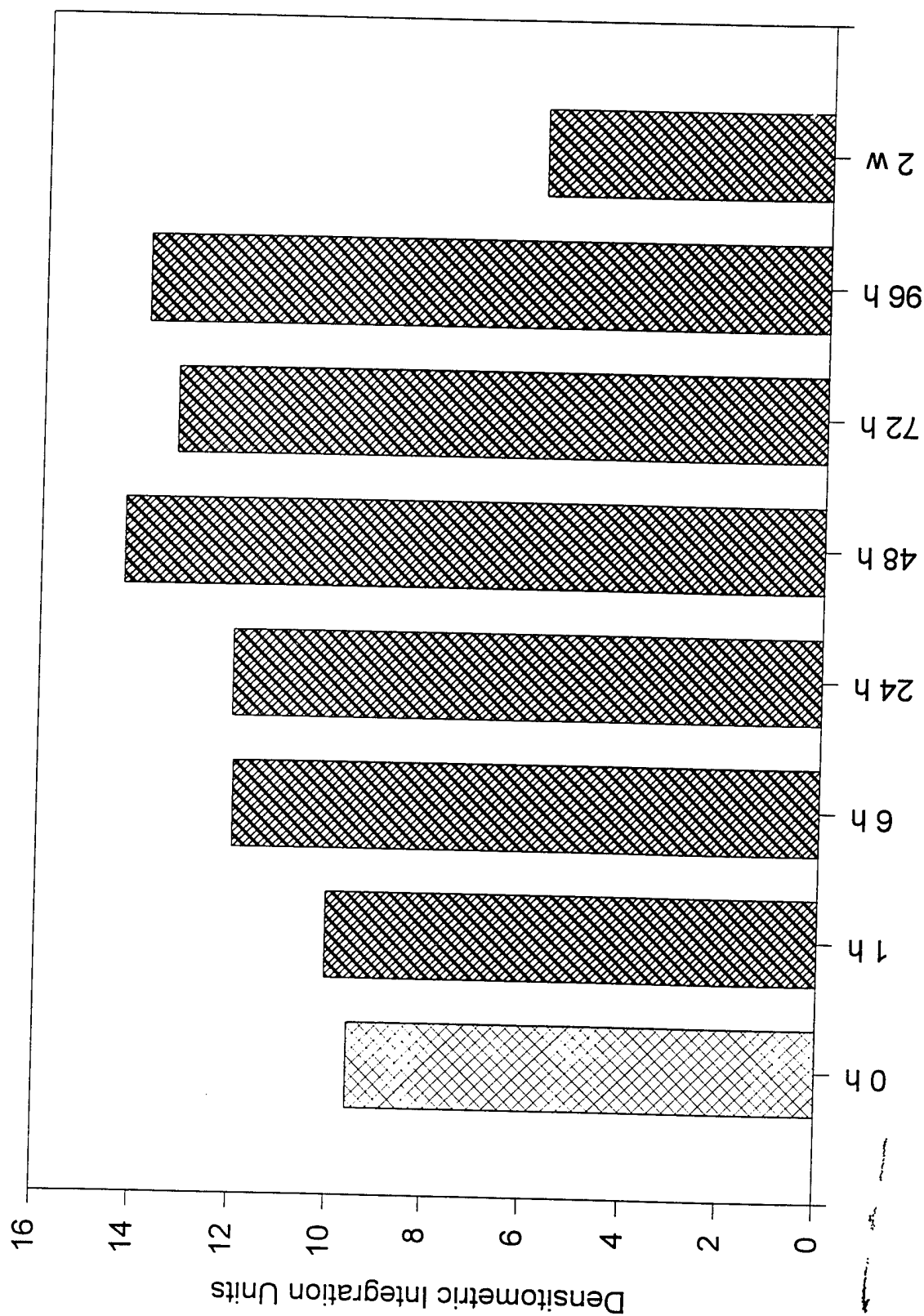
PKC-epsilon Levels in Patient #7 vs. Treatment with Bryostat-1



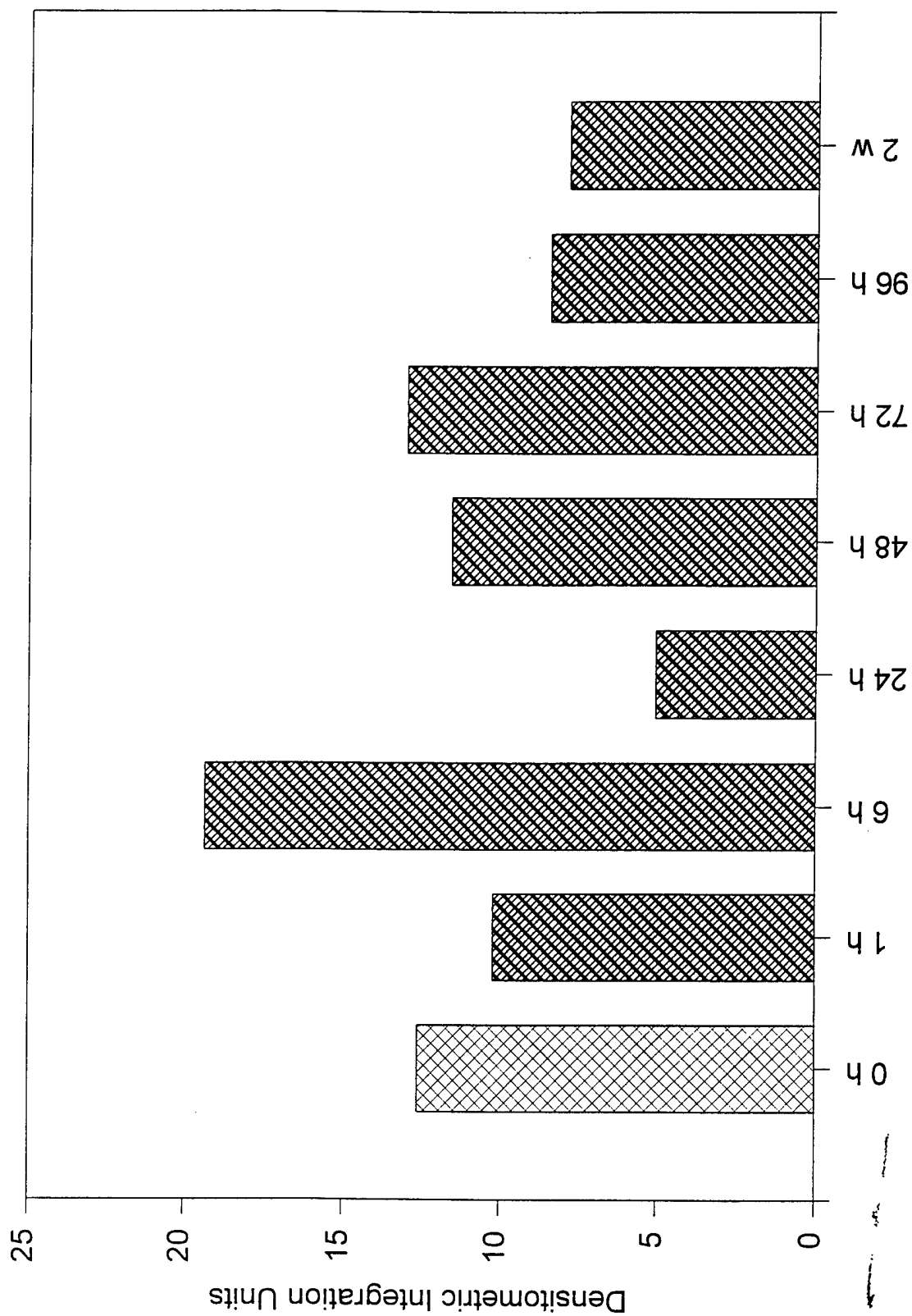
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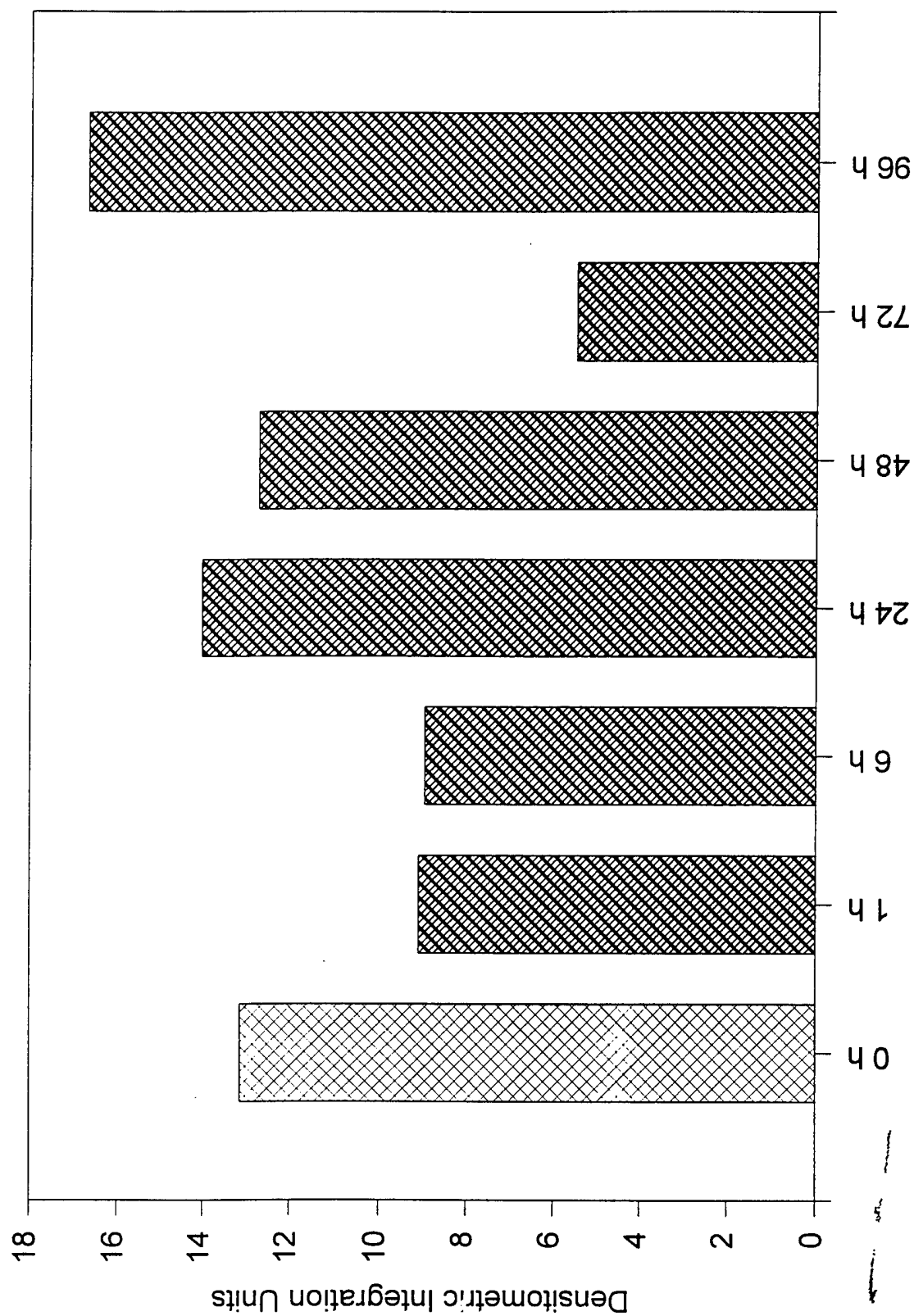
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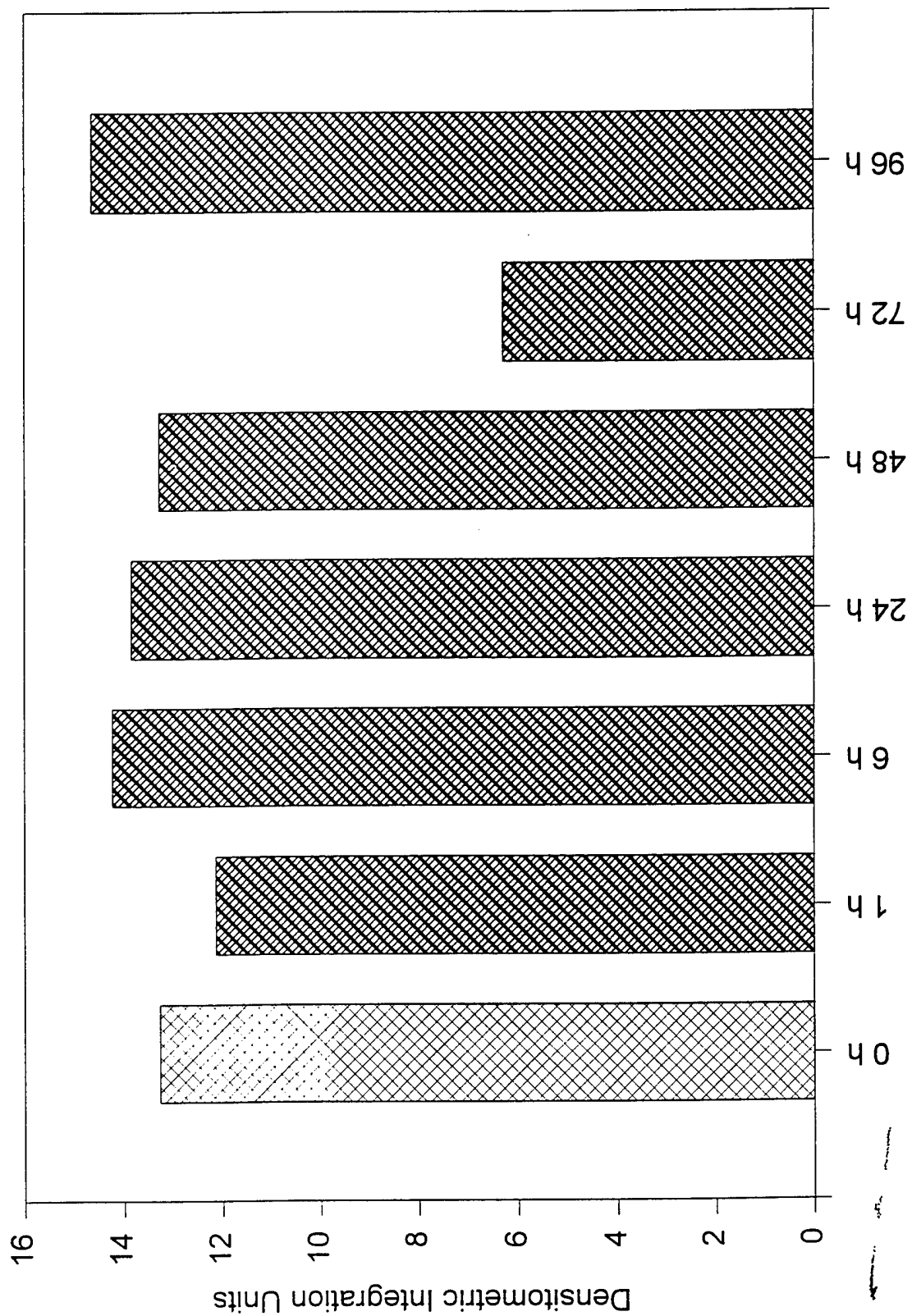
PKC- μ Levels in Patient #7 vs. Treatment with Bryostat-1



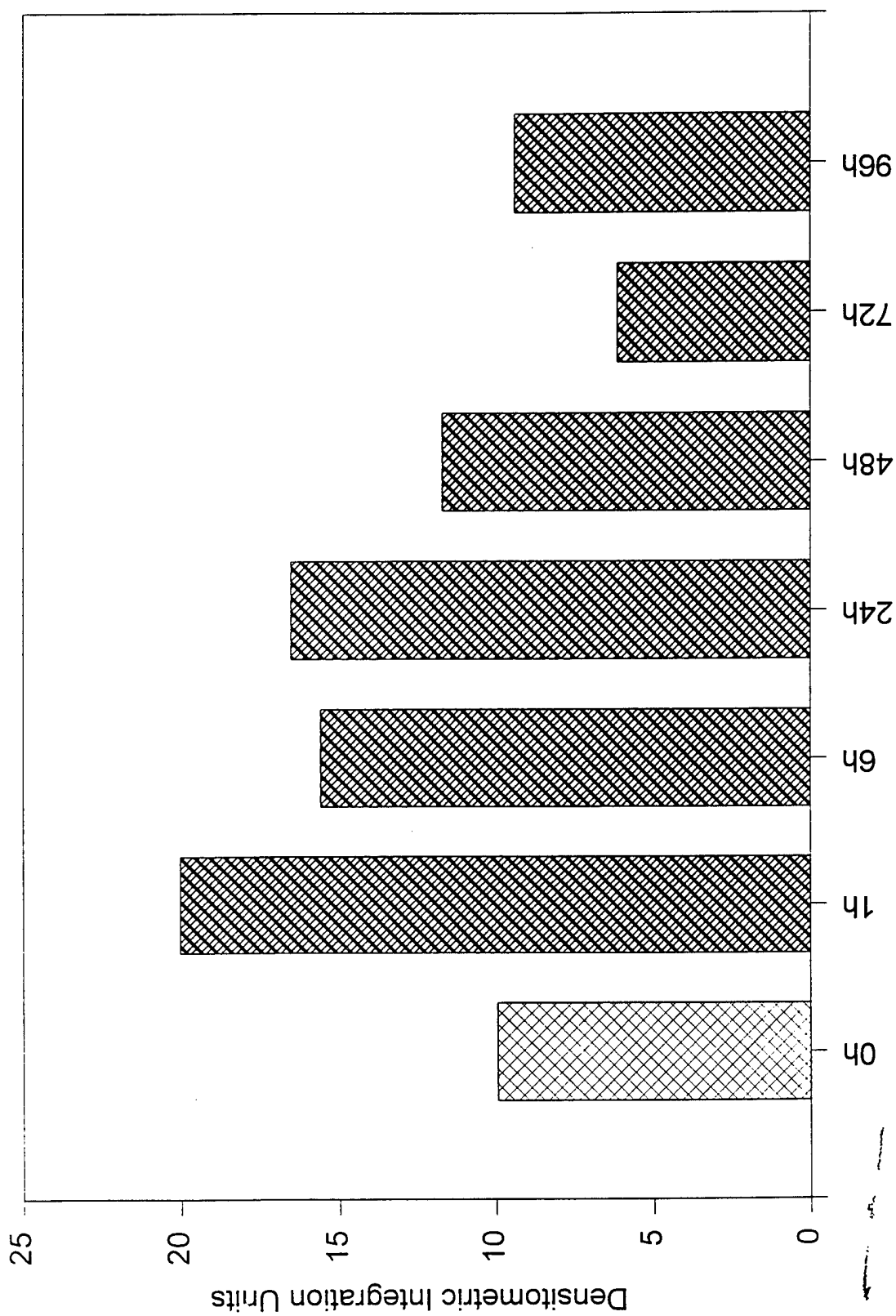
PKC-alpha Levels in Patient #8 vs. Treatment with Bryostatatin-1



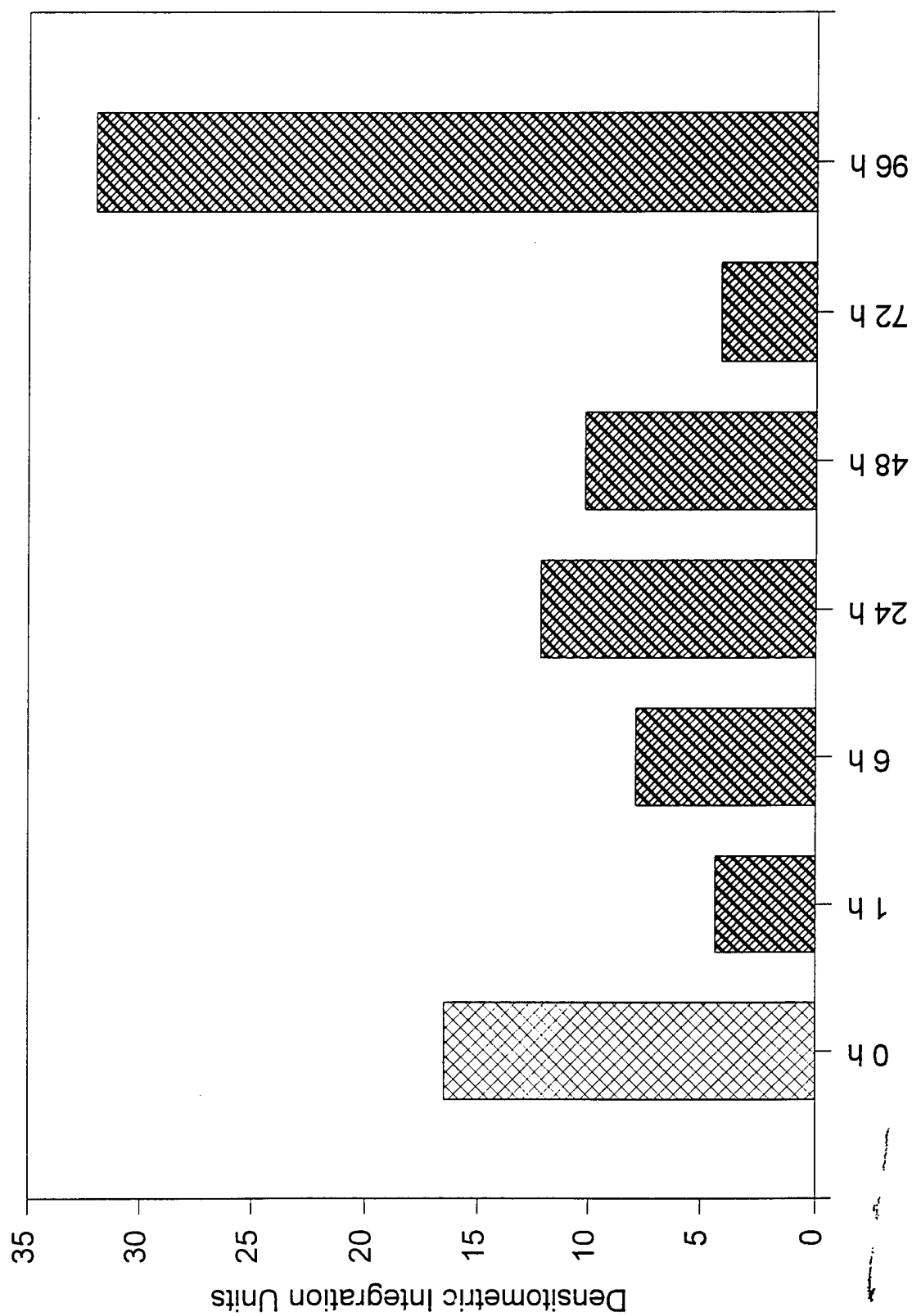
PKC-delta Levels in Patient #8 vs. Treatment with Bryostat-1



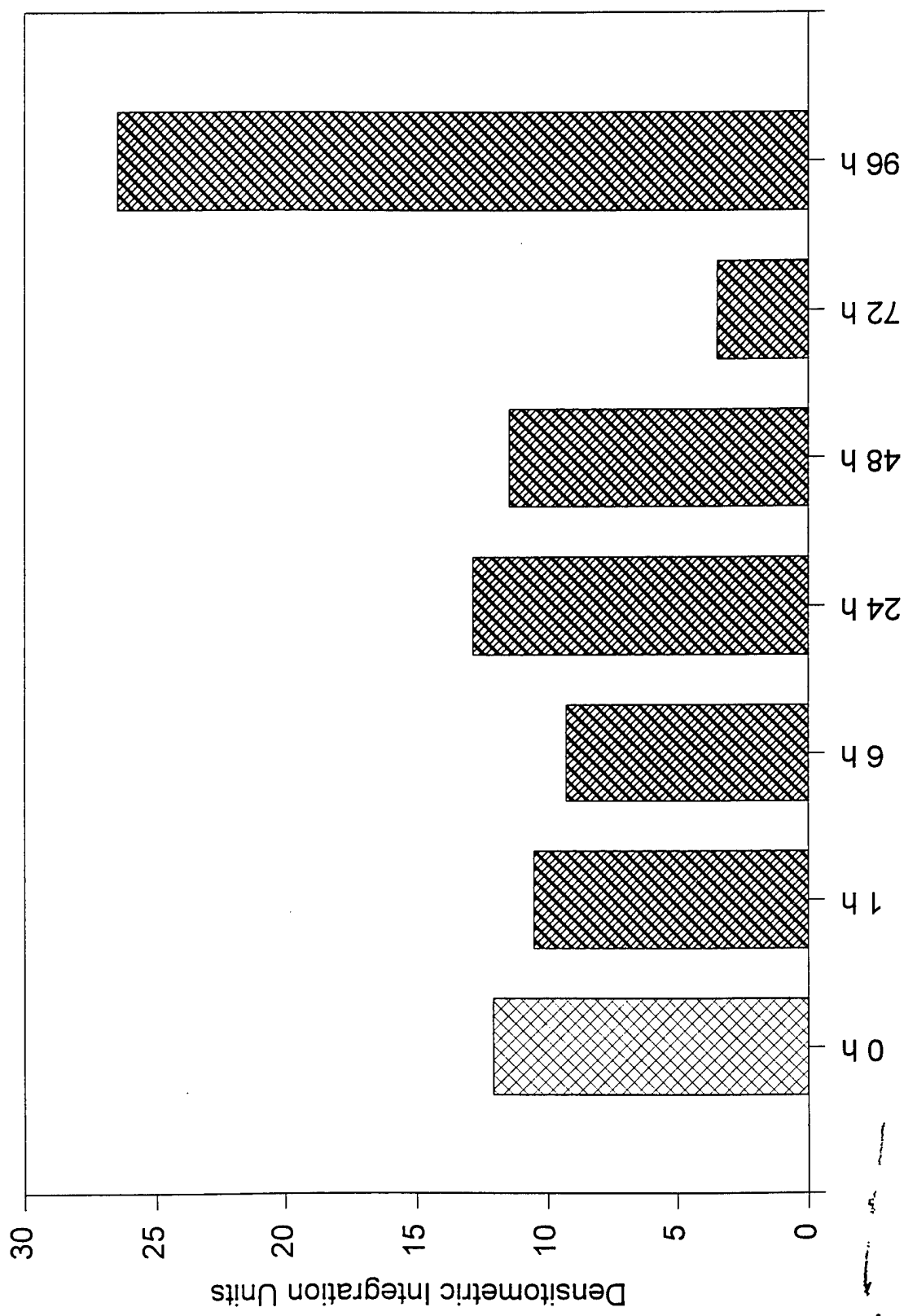
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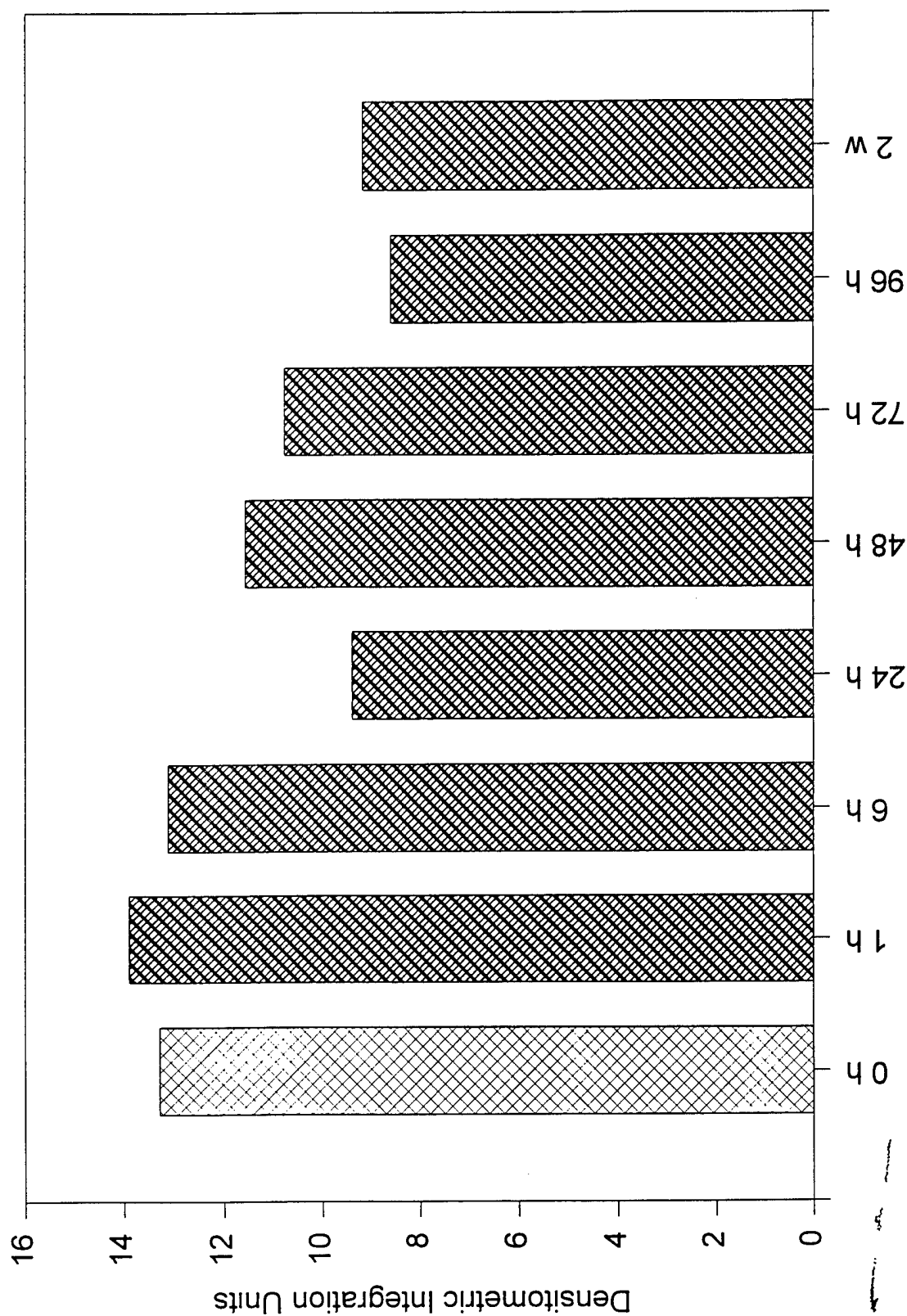
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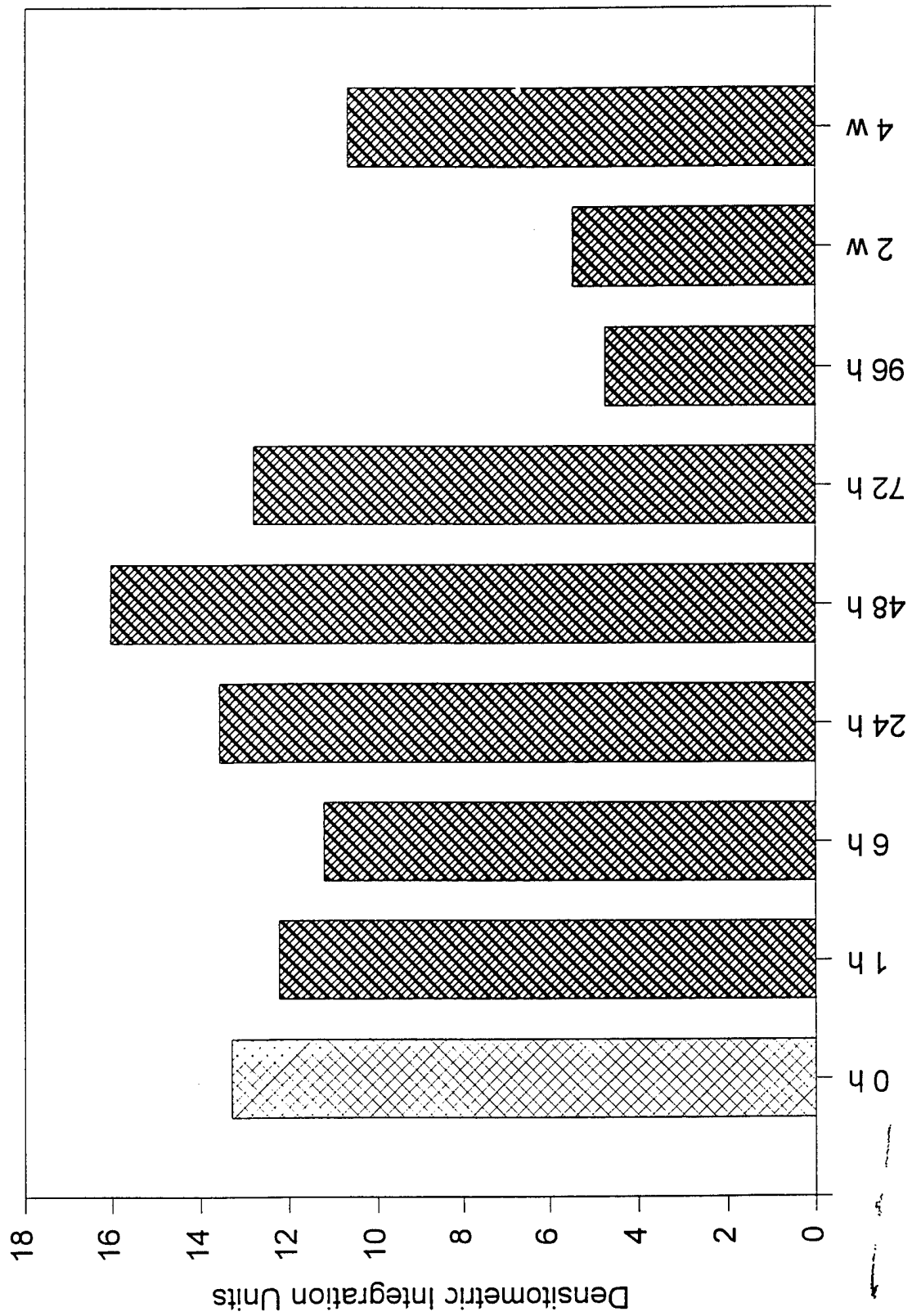
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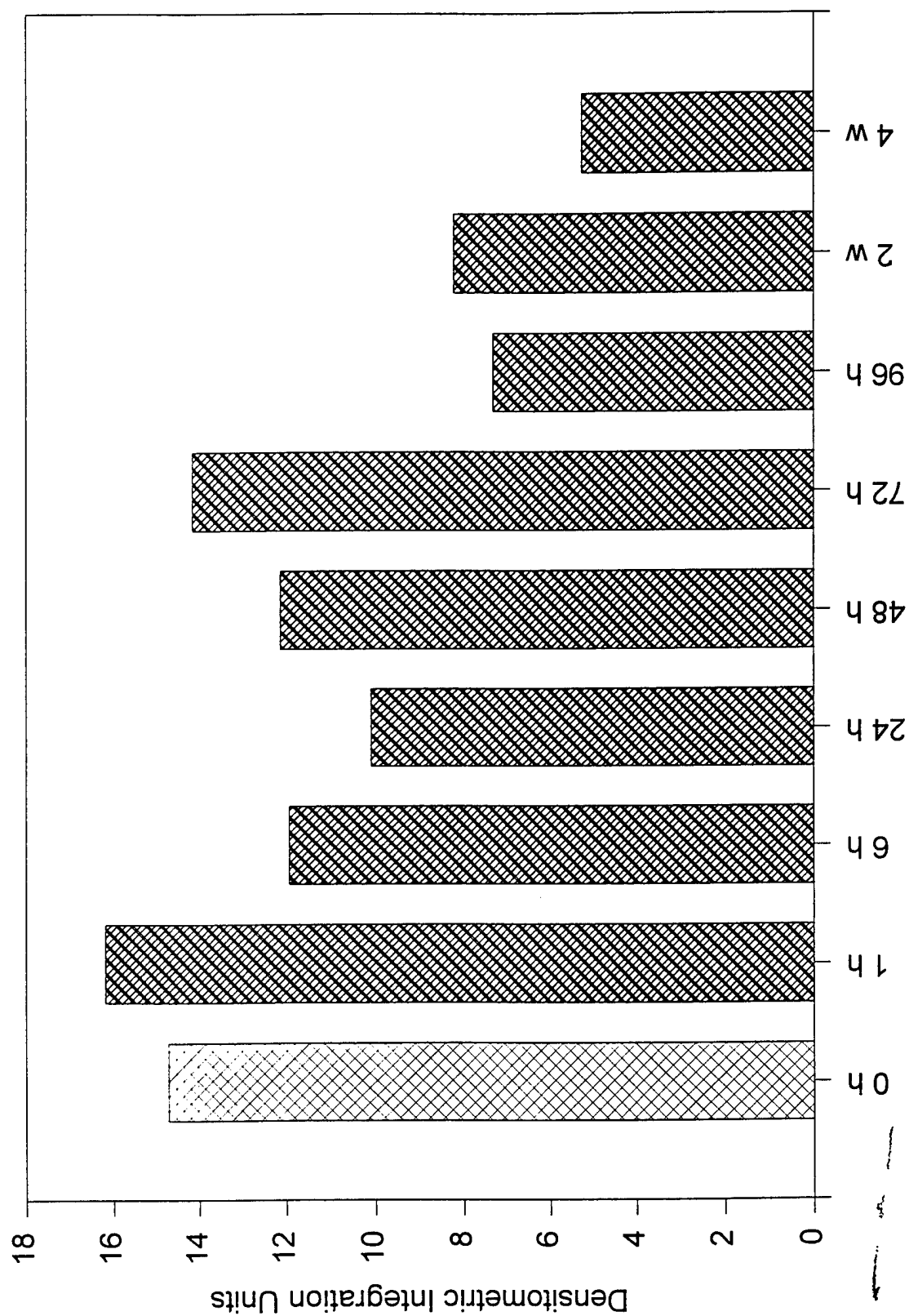
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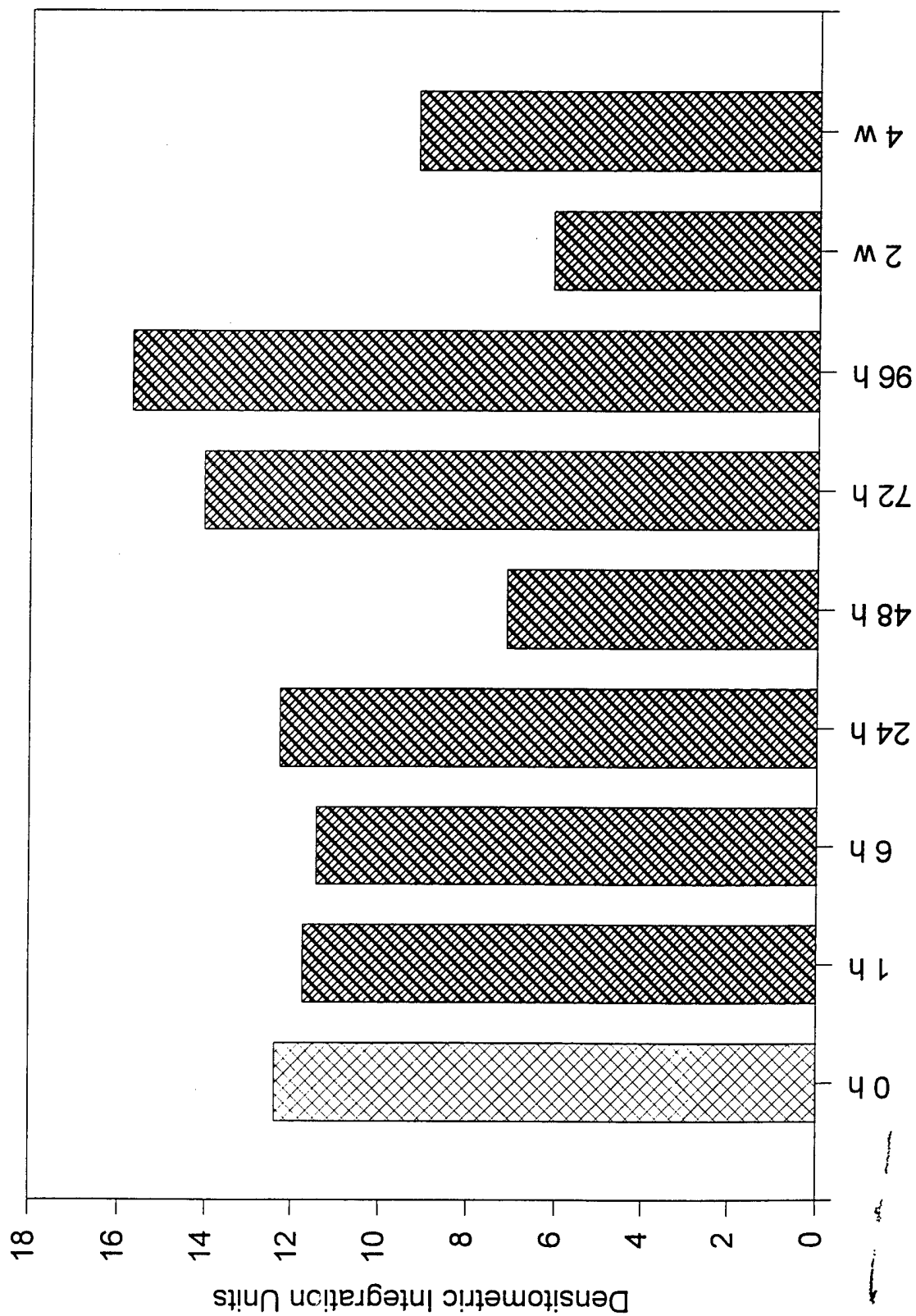
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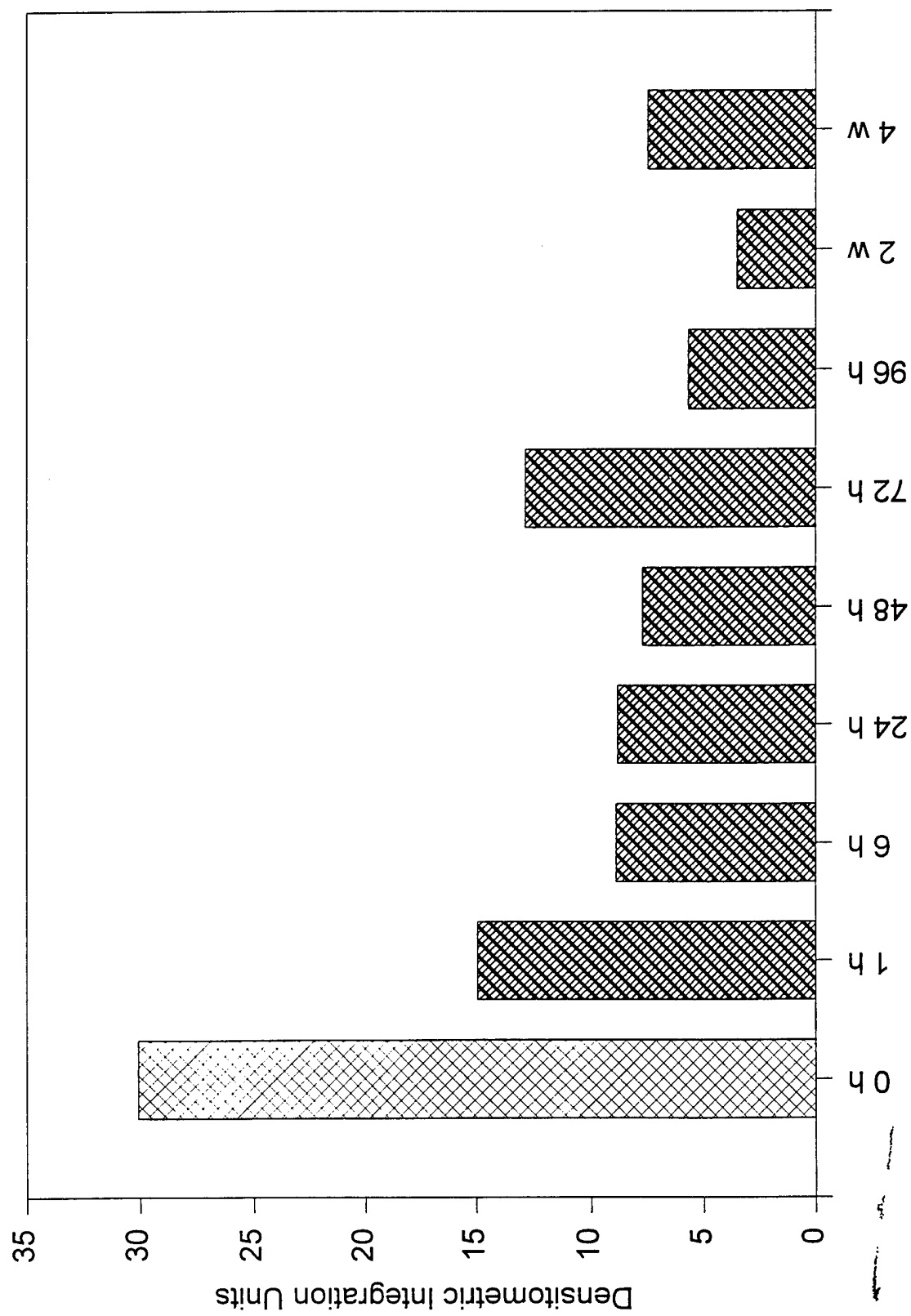
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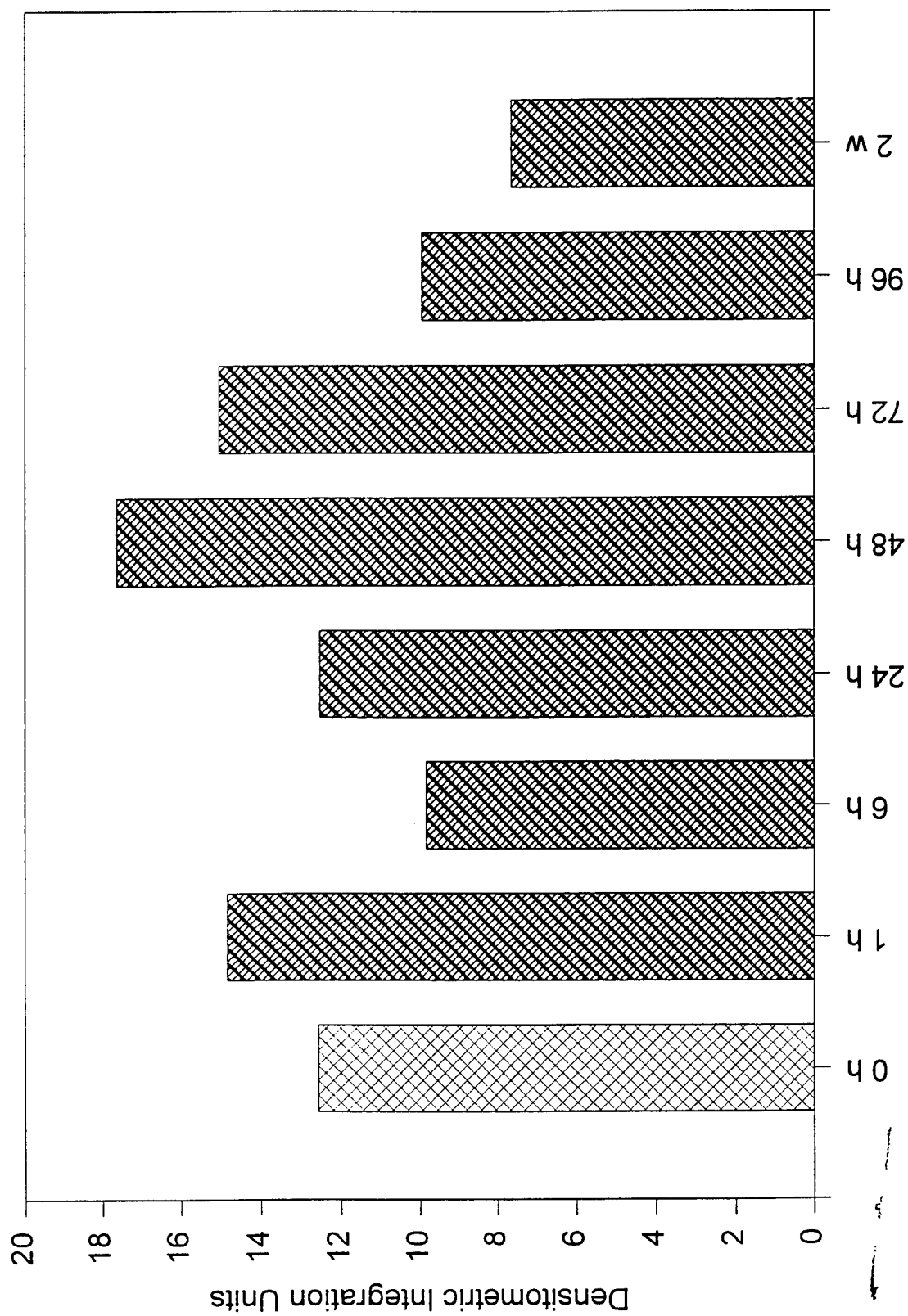
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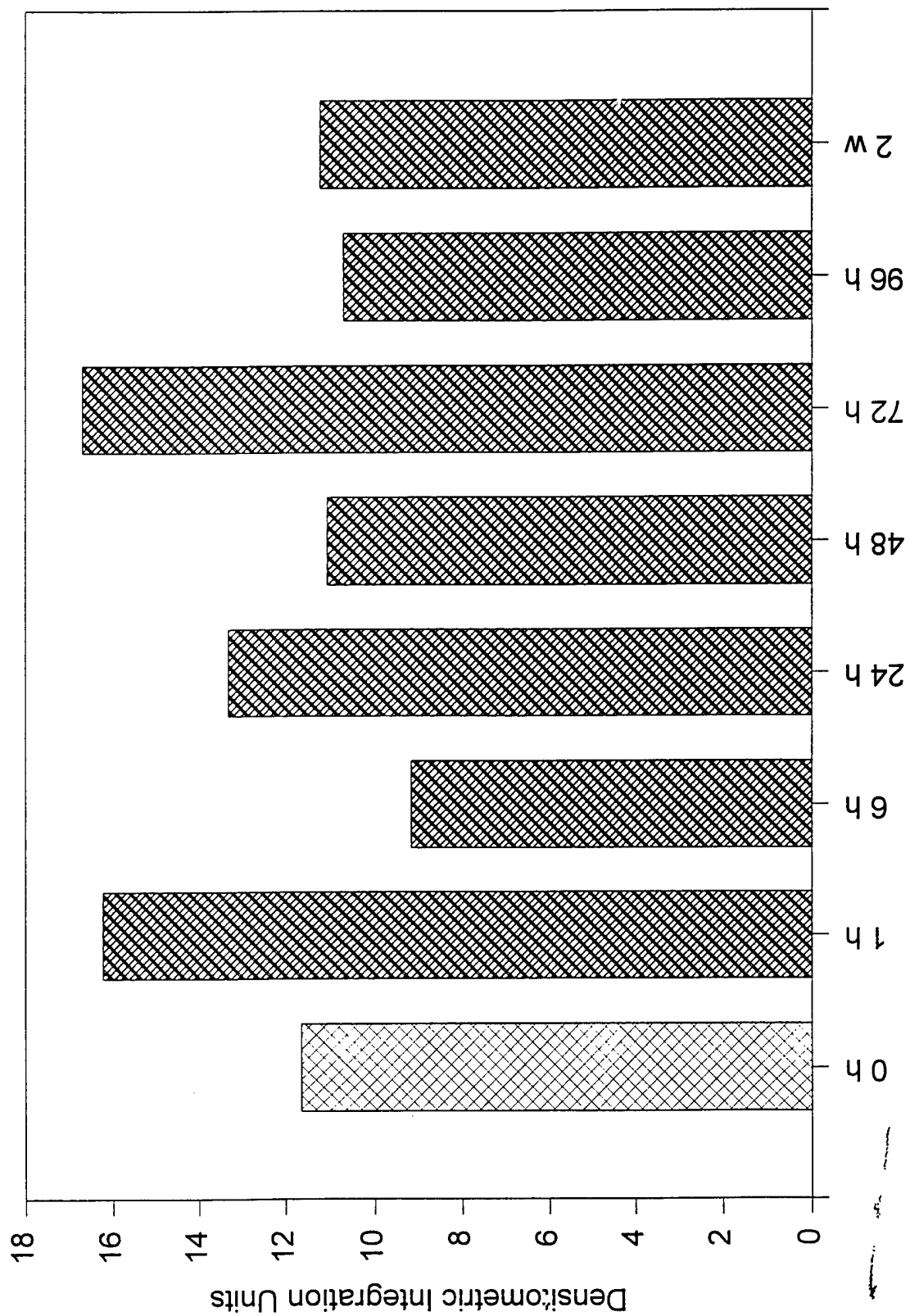
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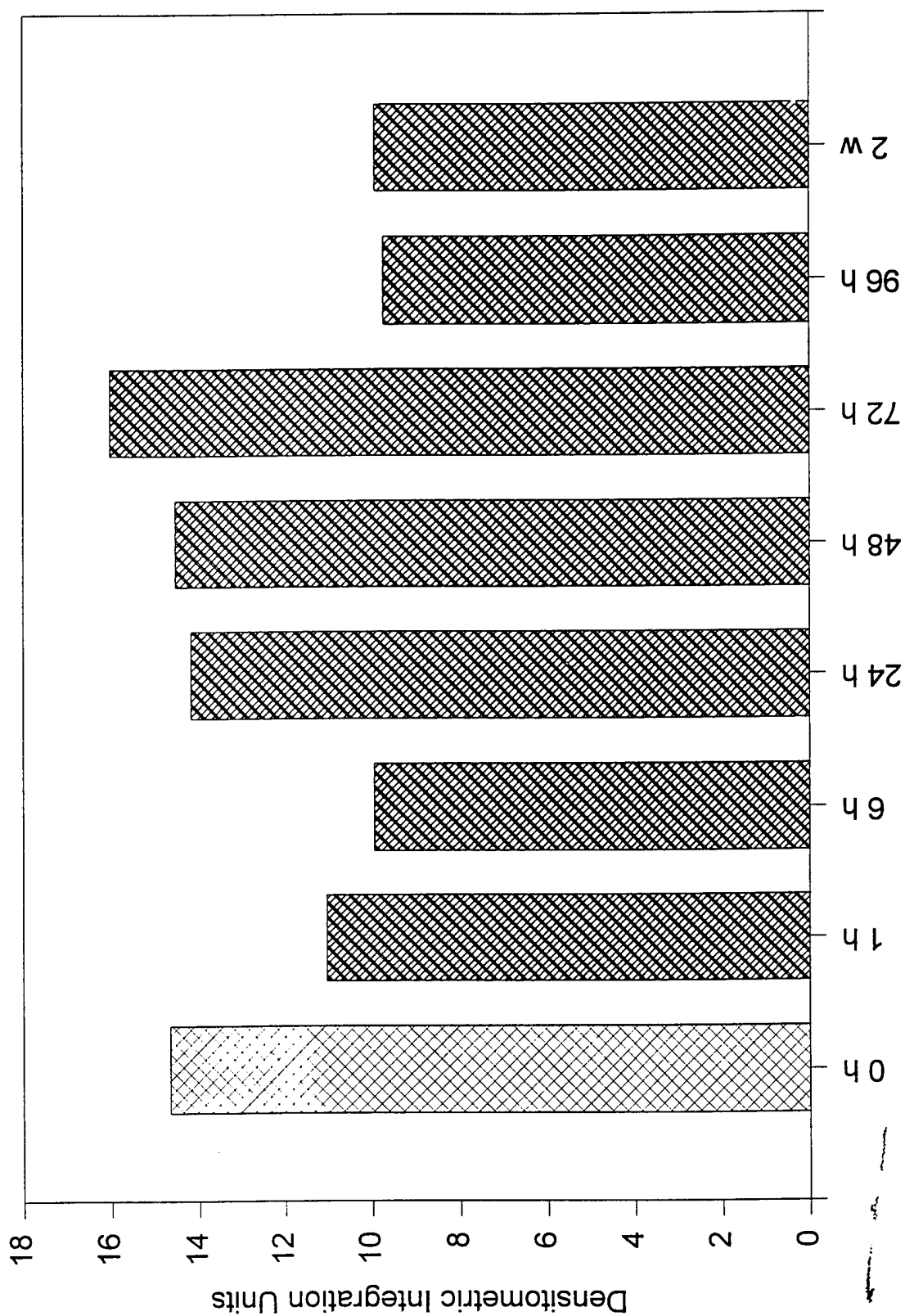
PKC-alpha Levels in Patient 10 vs. Treatment with Bryostat-1



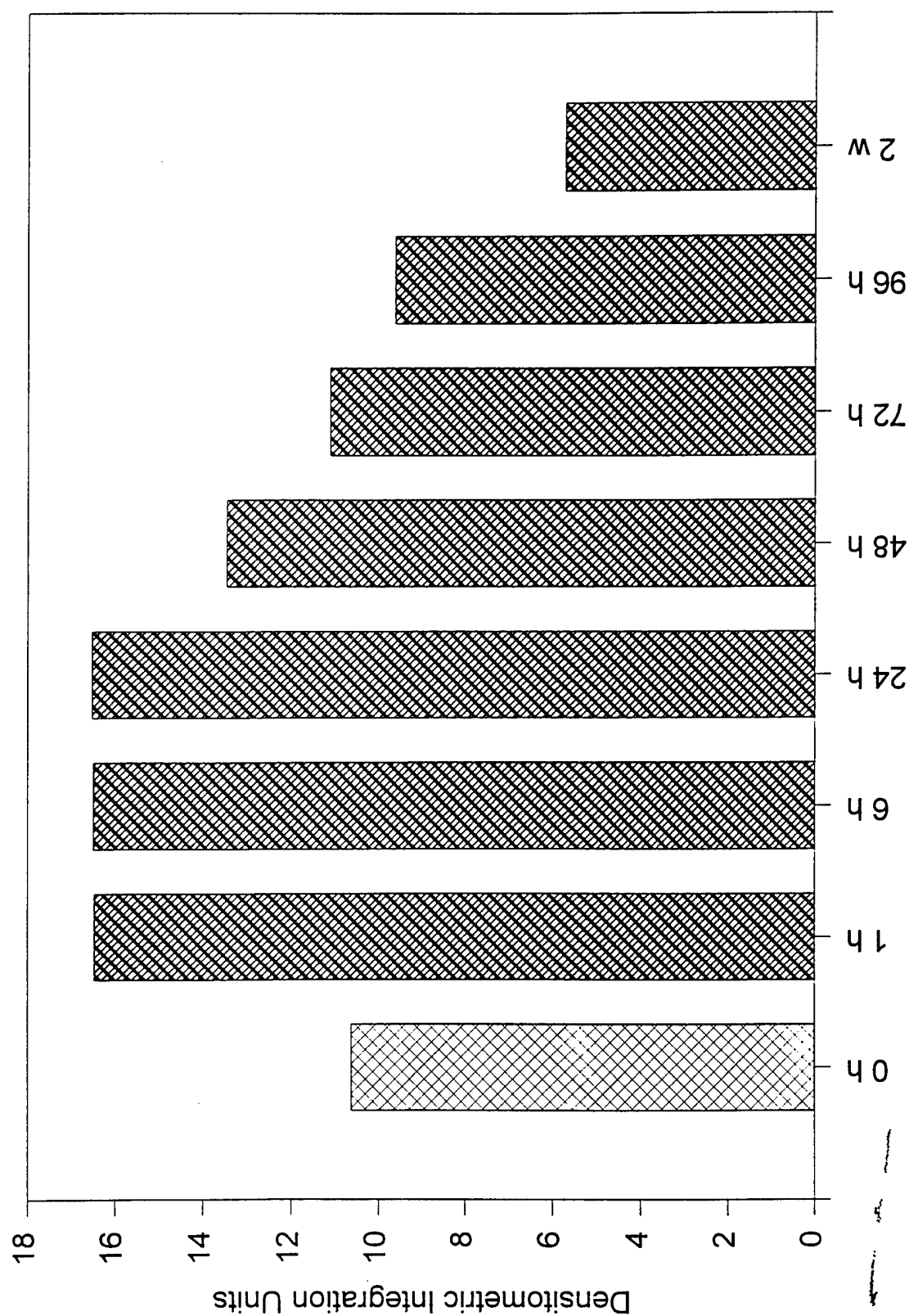
PKC-delta Levels in Patient #10 vs. Treatment with Bryostat-1



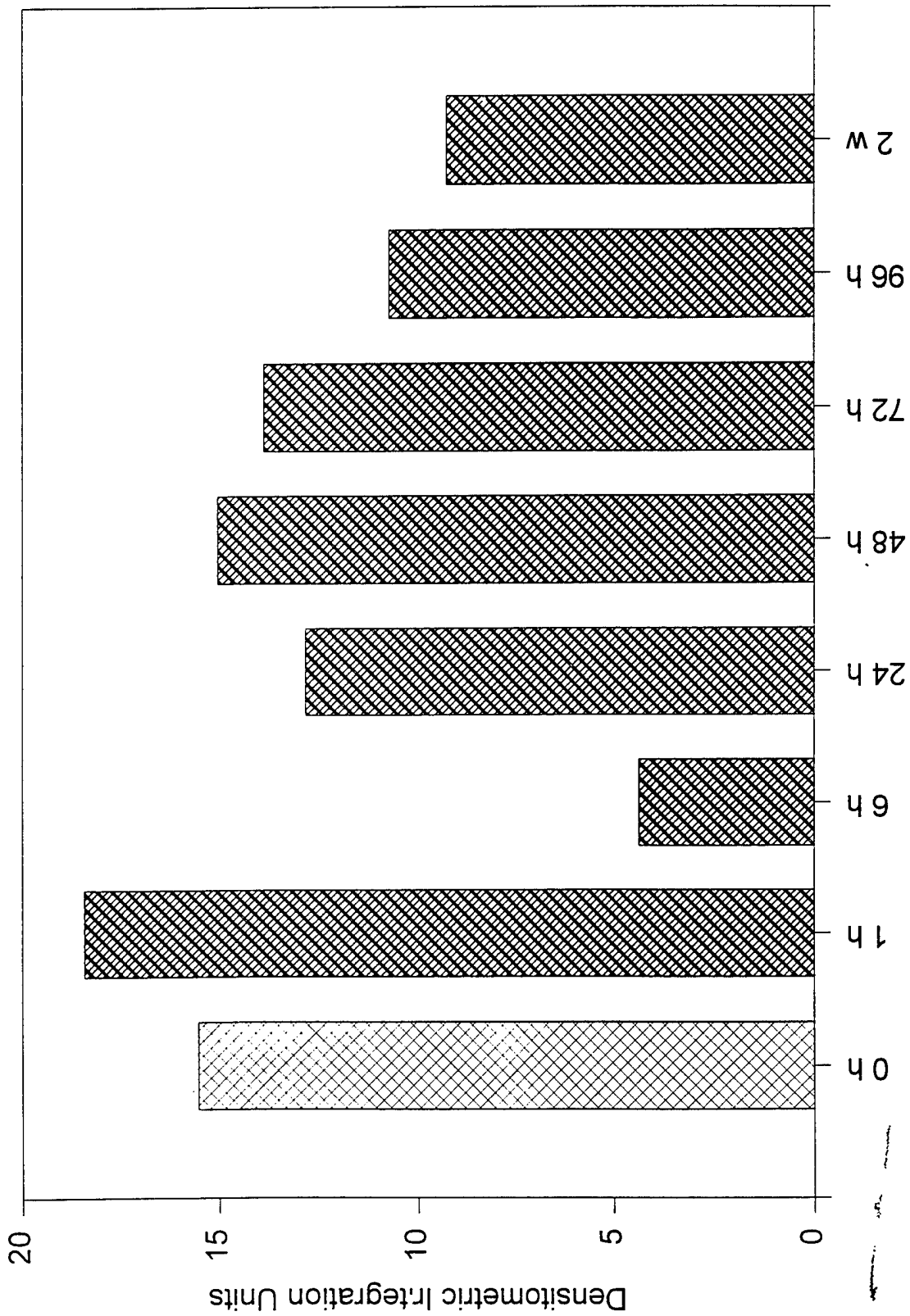
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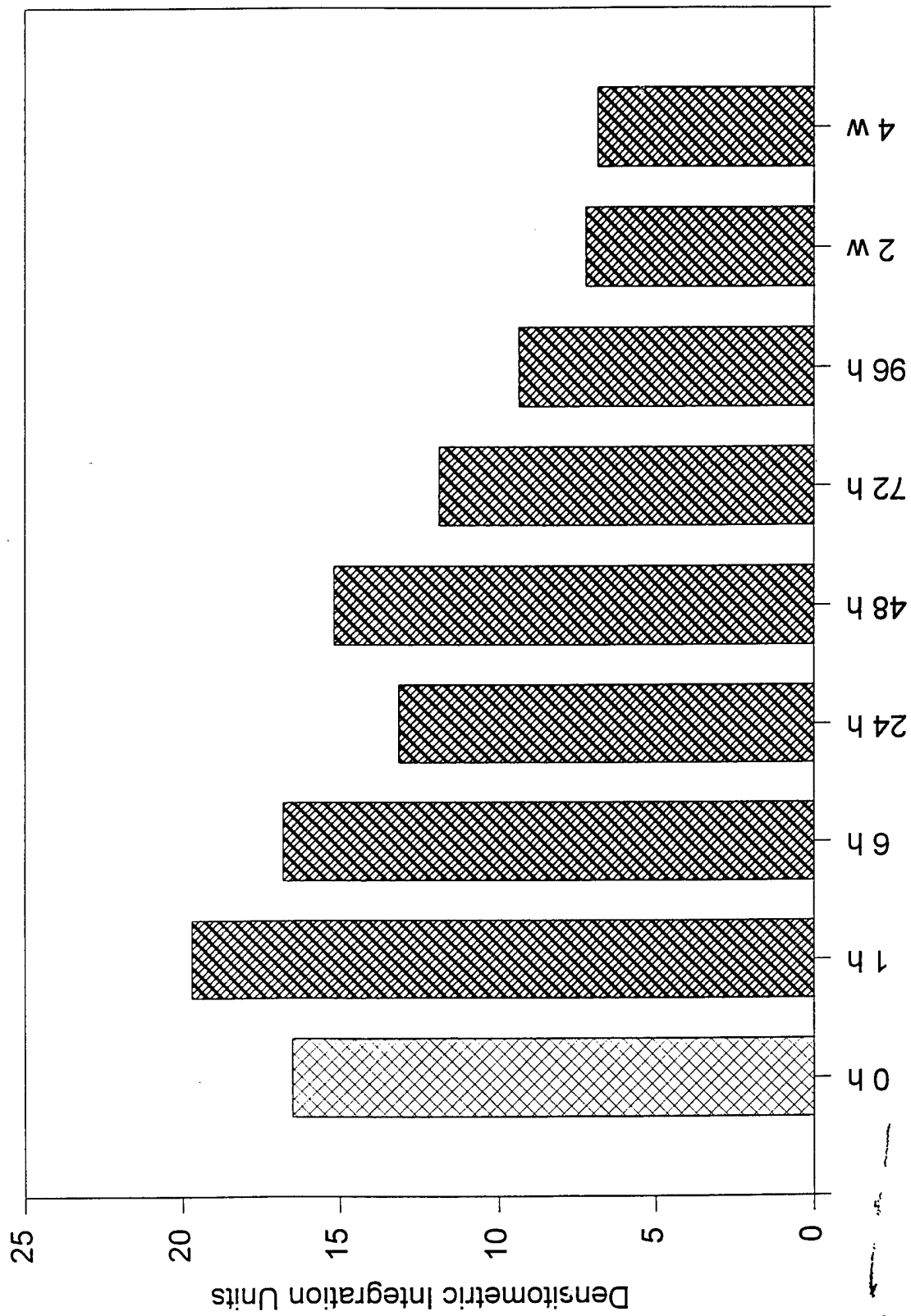
PKC-eta Levels in Patient #10 vs. Treatment with Bryostat-1



PKC-gamma Levels in Patient #10 vs. Treatment with Bryostatin-1

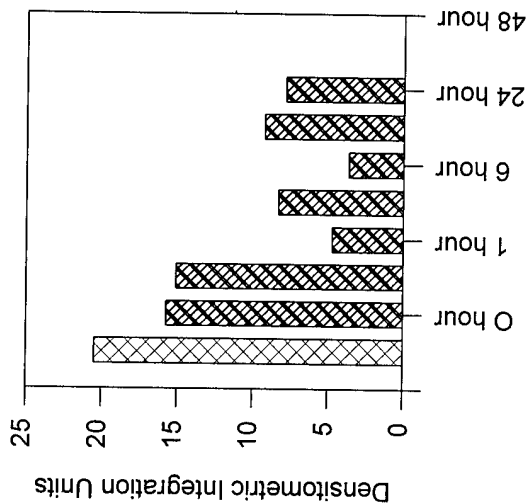


PKC- μ Levels in Patient #10 vs. Treatment with Bryostat-1

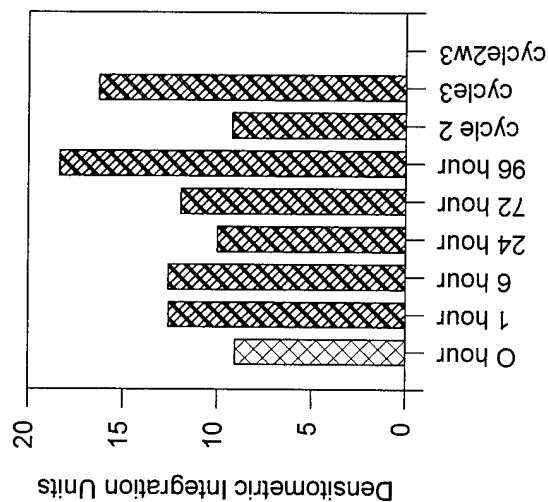


Patient #111: Protein Kinase C Levels vs. Treatment with Bryostat-1

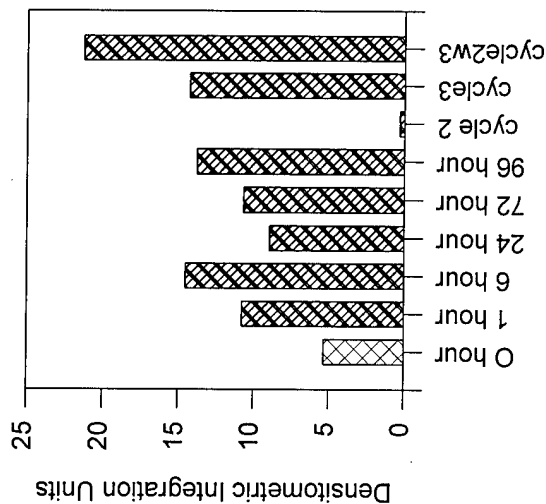
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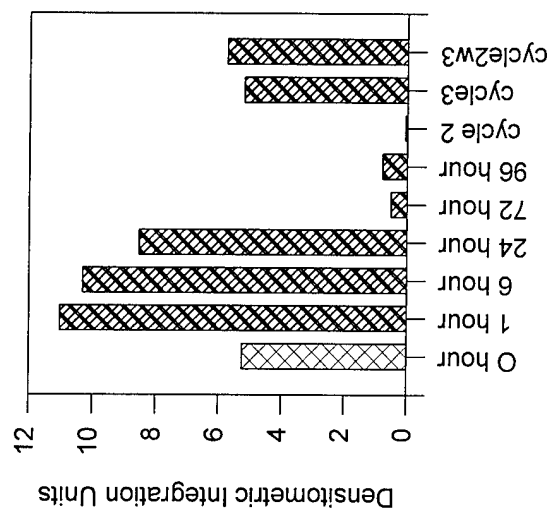
PKC-alpha



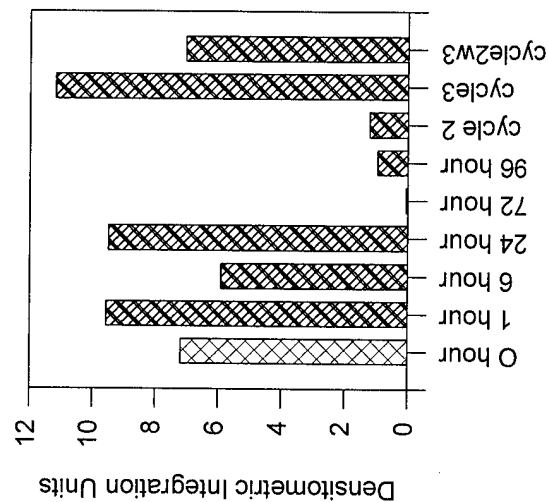
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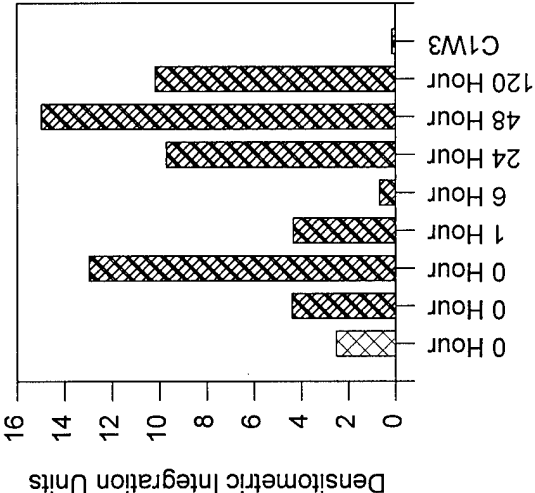
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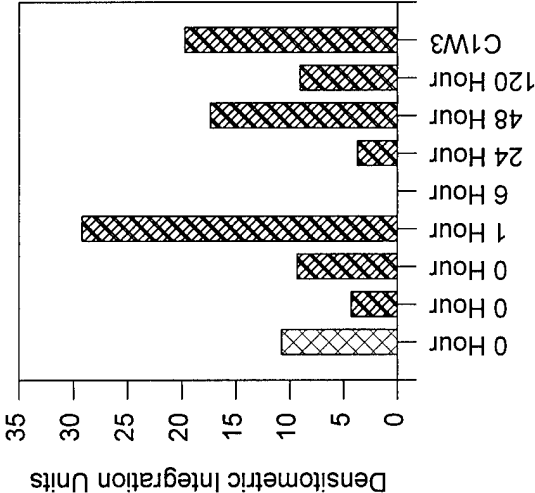
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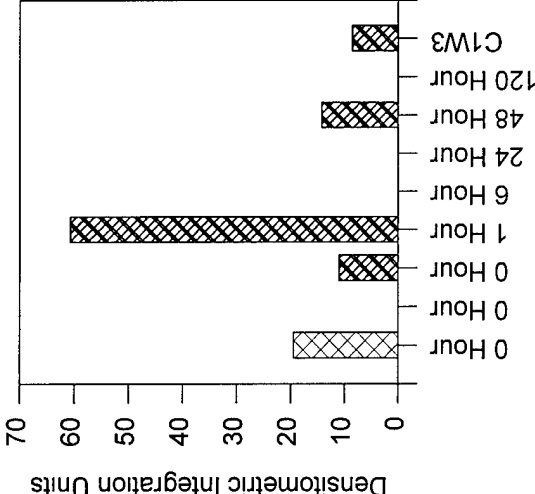
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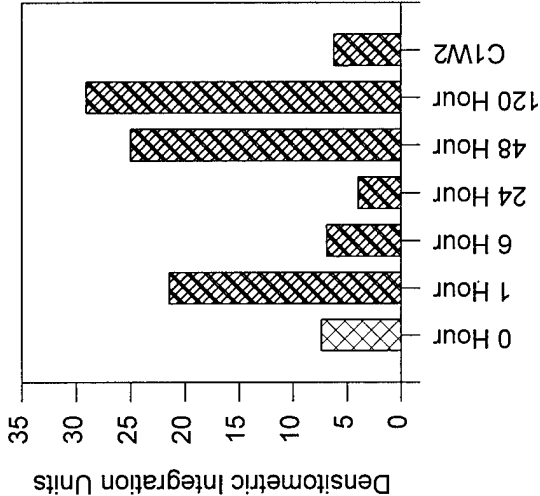
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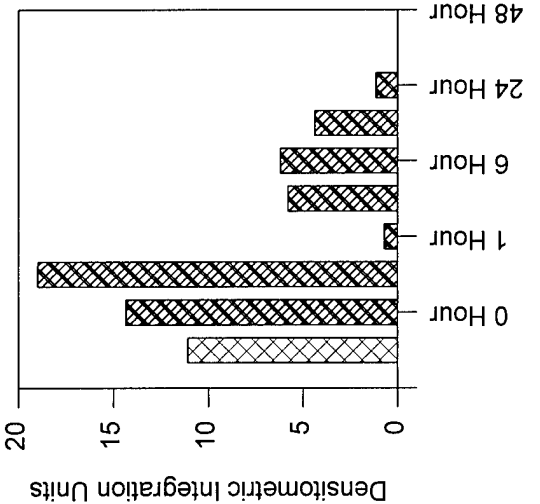
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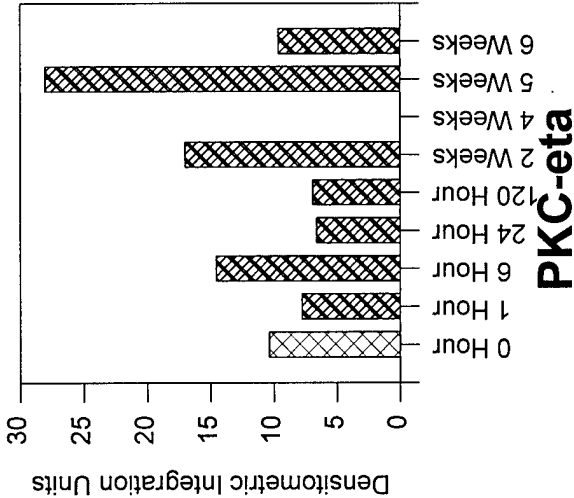
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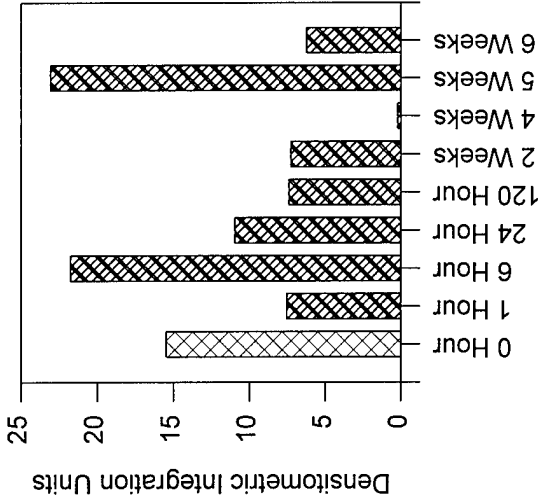
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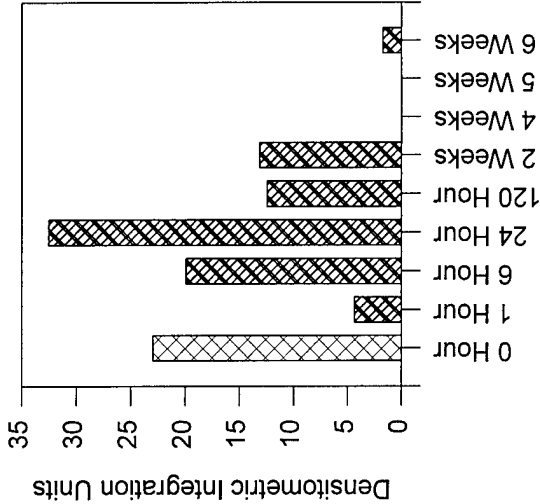
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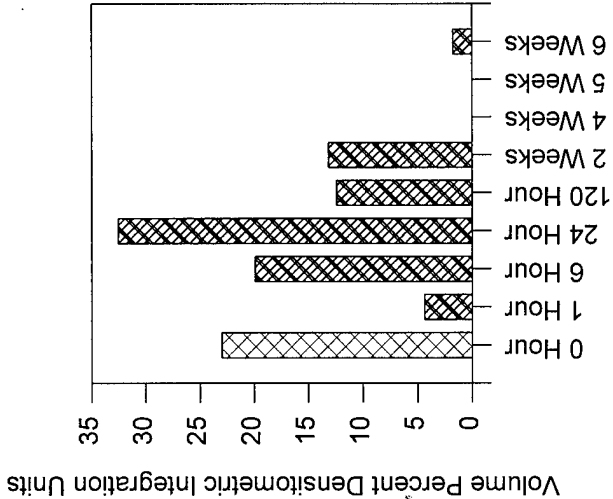
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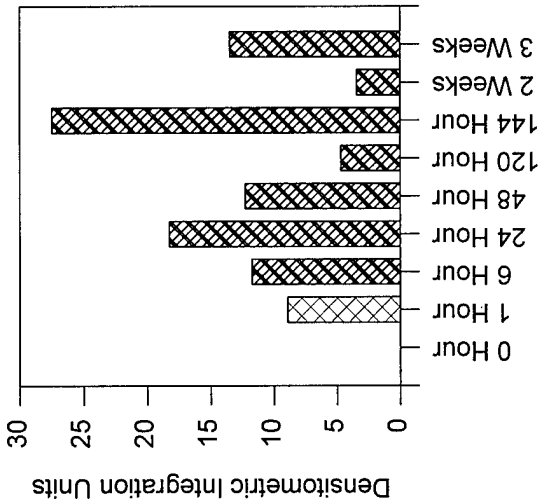
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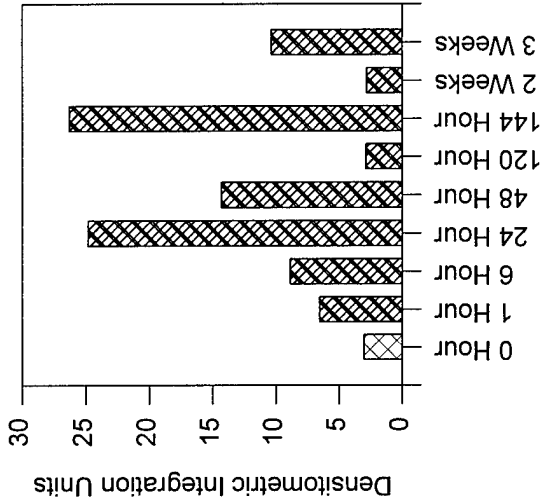
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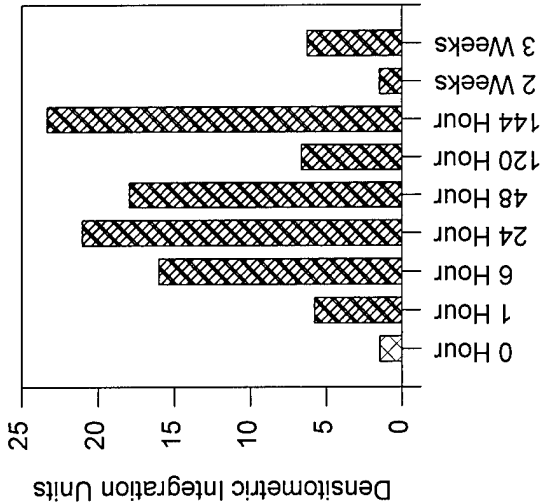
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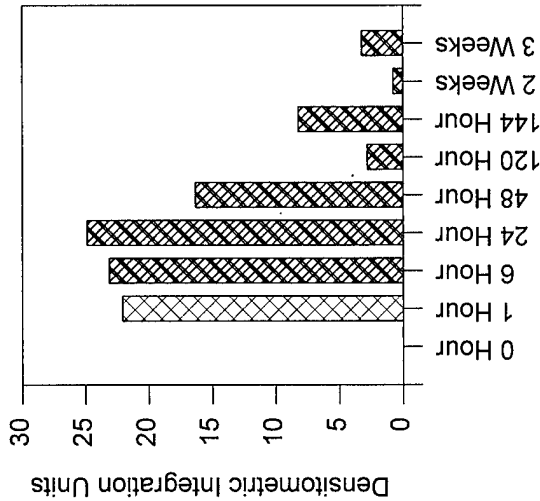
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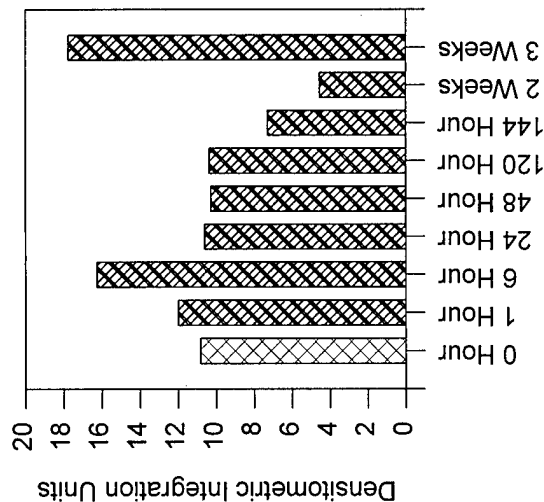
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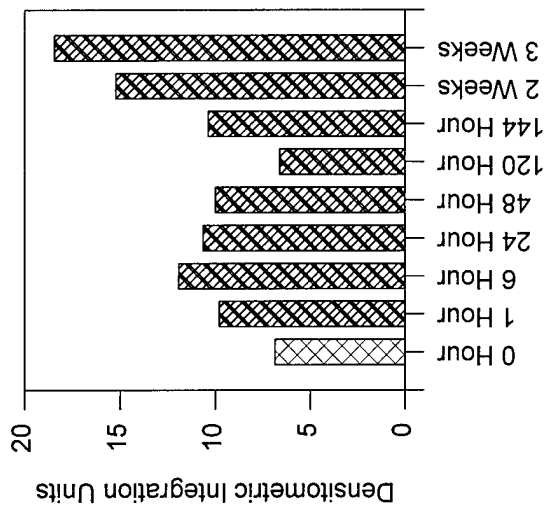
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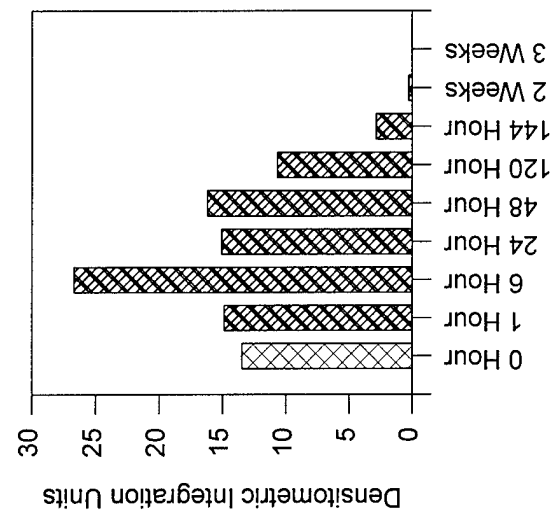
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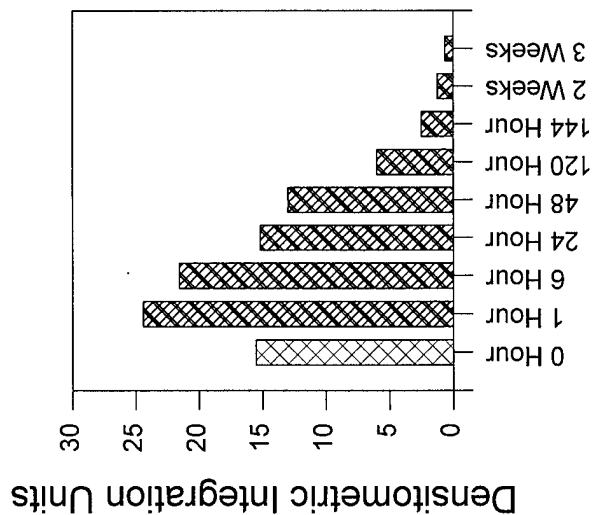
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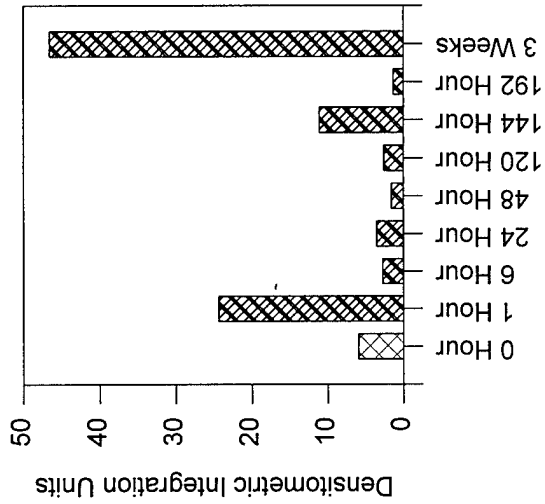
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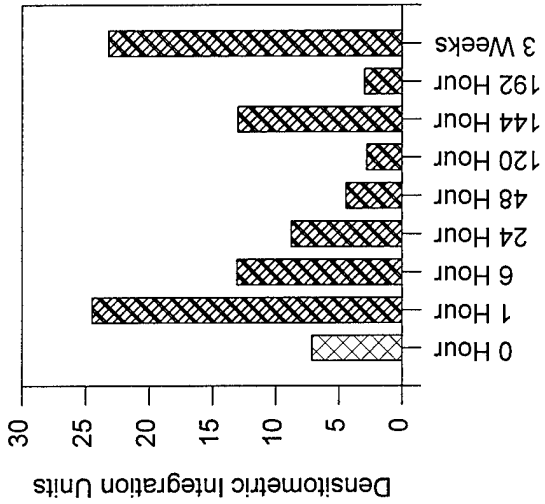
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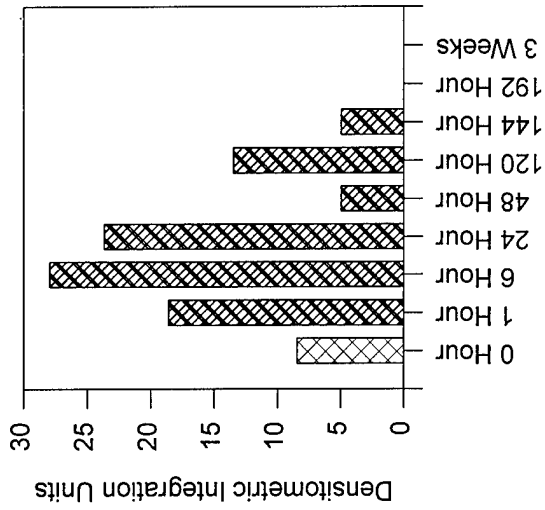
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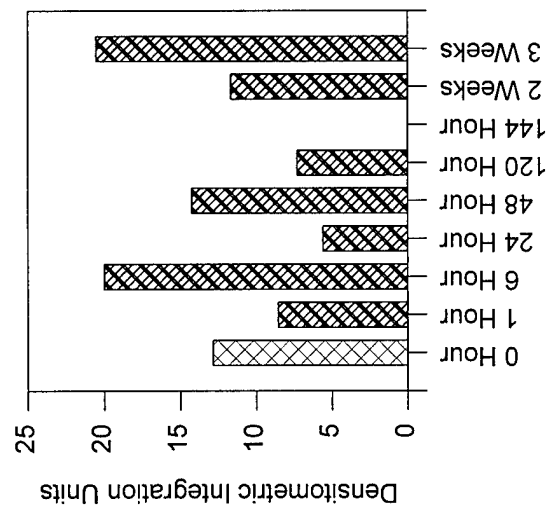
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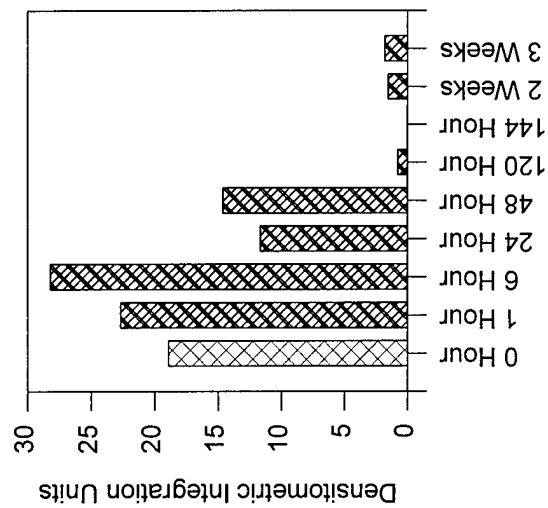
PKC-eta



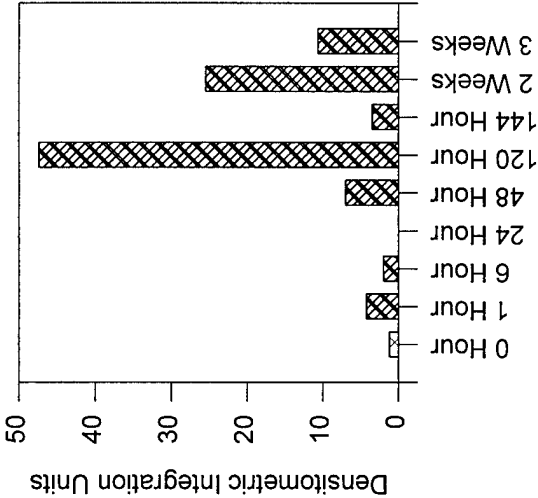
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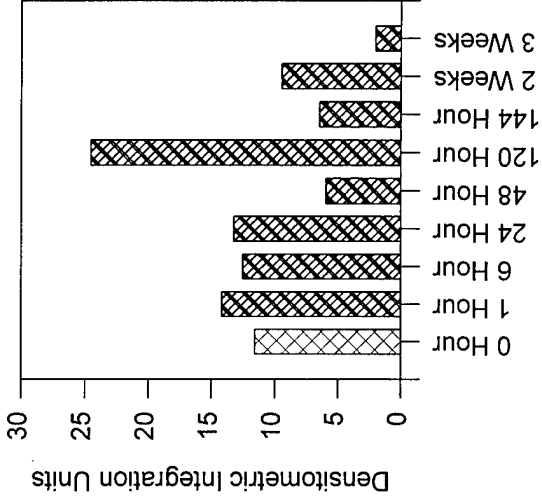
PKC-eta



PKC-alpha



PKC-delta



PKC-eta

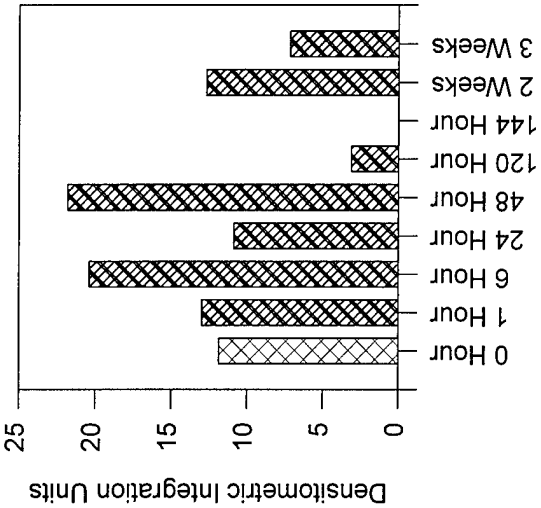
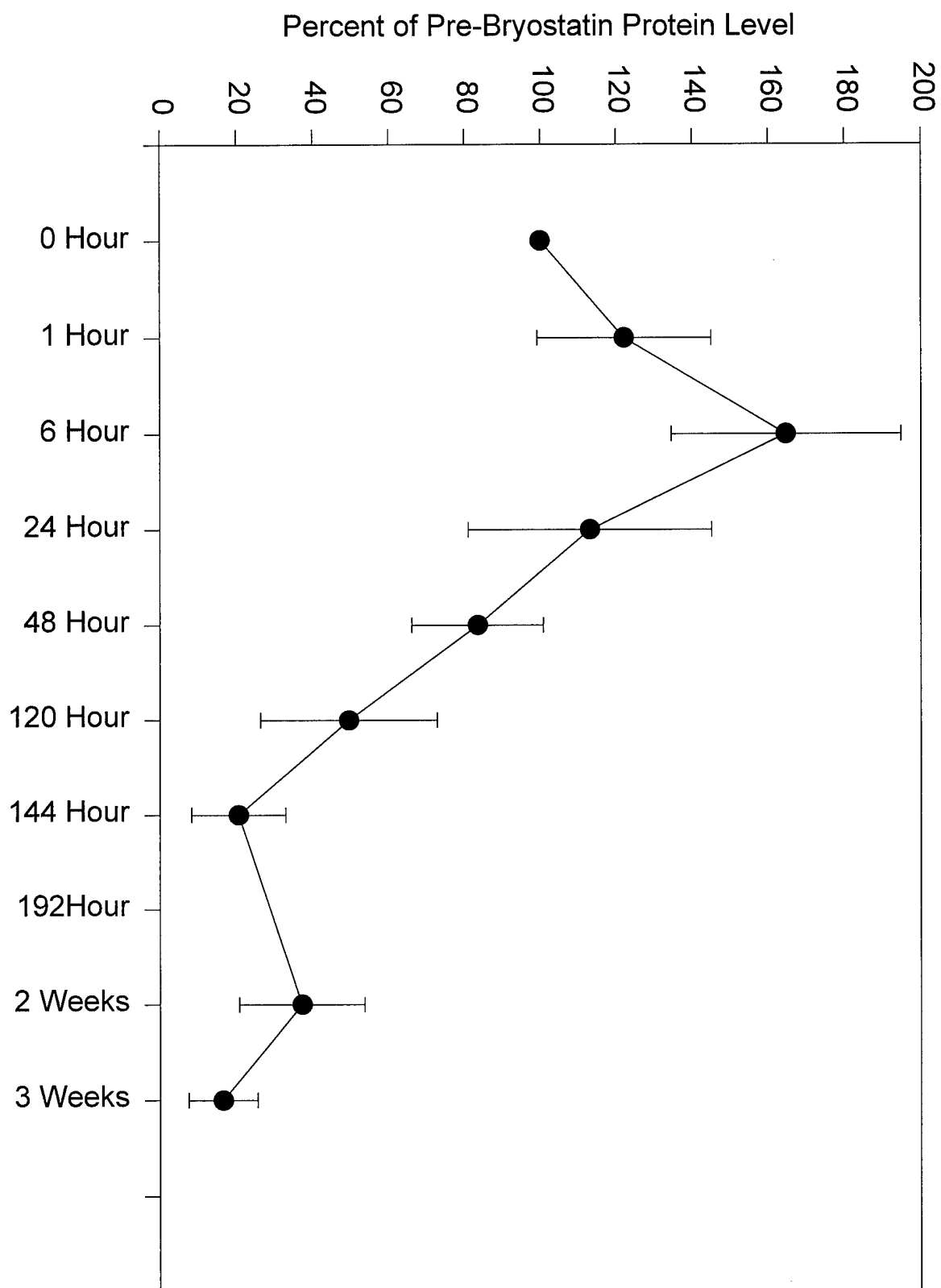


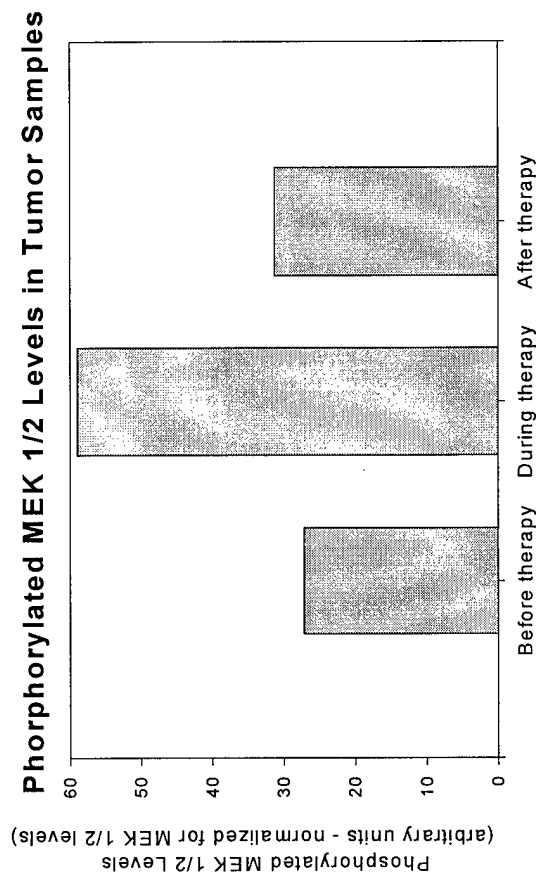
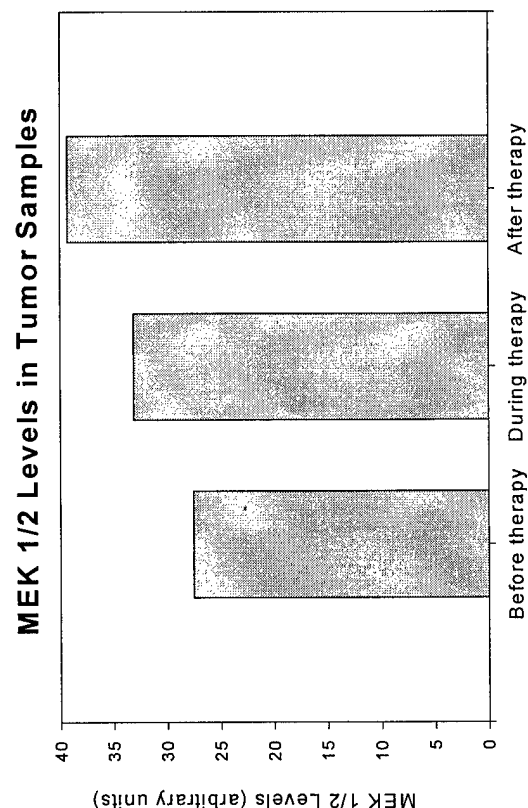
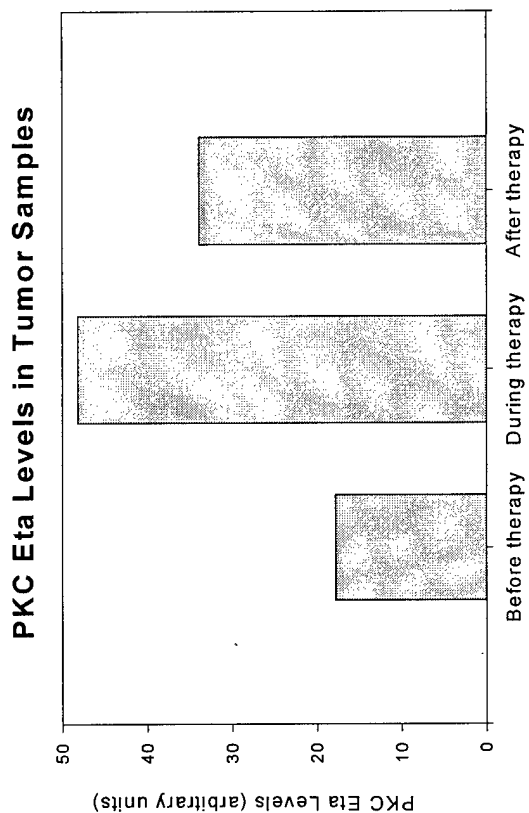
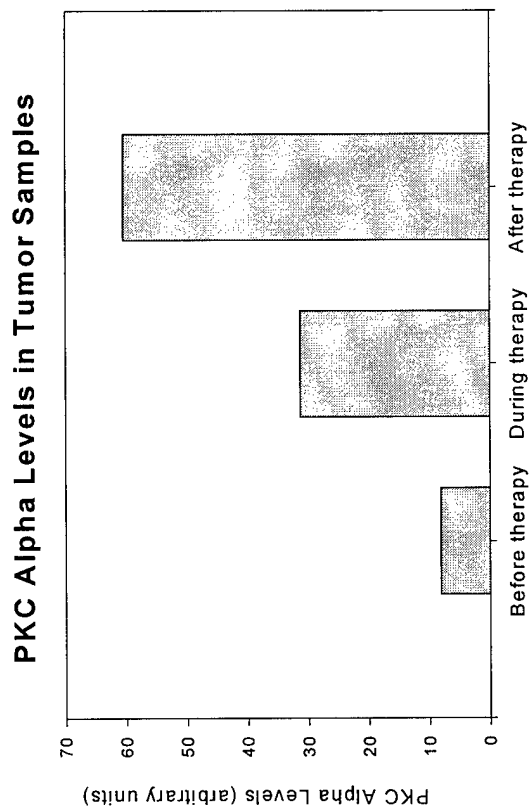
Fig 6

Mean PKC-eta Levels for Patients 16-19, 22-24



Phase 1 Trial of Bryostatin-1:

Serial Biopsy of Tumor From Patient 11 Treated with Bryostatin-1



Inhibition of PKC-alpha Levels by Anti-sense Therapy

Bryostatin-1 is a natural product therapeutic agent and is, therefore, likely to have additional mechanism of action to its central effects on PKC levels and activity. With this in mind, we also decided to study the effects of a much more specific therapeutic intervention: the suppression of PKC alpha expression by therapy with a synthetic anti-sense molecule.

Background.

Anti-sense oligonucleotide action.

Ever since the development of the Central Dogma regarding the transmission of genetic information from the DNA via messenger RNA leading to the synthesis of a protein, it has been apparent that the possibility existed to interrupt this process specifically by blocking the translation of the mRNA molecule. Though there are several way in which this has now been achieved, the most common is through the use of anti-sense oligonucleotides. These consist of small pieces of single stranded DNA that have a sequence that is complimentary to the sequence of a portion of the mRNA that it is being targeted. On binding the anti-sense oligonucleotide blocks mRNA translation in two ways. Firstly, simple by steric hindrance: the translation machinery is blocked on the message. Secondly, the mRNA bound to the oligonucleotide is a substrate for elimination by RNase H. It is thought by many that this second mechanism is the most important and in this way the oligonucleotide, which is unaffected by RNase H can act in a catalytic mode, with one molecule targeting the destruction of many RNA molecules. While, simple DNA based oligonucleotides do indeed work well, they can be degraded rapidly by nucleases and so have a rather short half-life *in vivo* and so various modified molecules have been used. One of the modifications that has been used a lot is the phosphorothioate oligonucleotide which has much better stability. Though the mechanism is still somewhat of a mystery, intravenously injected phosphorothioate oligonucleotides are taken up quite well by tissues, and so the delivery of these drugs is quite simple.

Anti-sense PKC-alpha oligonucleotide clinical trial.

A proprietary anti-sense oligonucleotide directed against PKC-alpha was about to be tested in early clinical trials in the DT program at the Lombardi Cancer Center, and so we decided that this would provide the perfect opportunity to examine the effect of the inhibition of PKC-alpha expression on cellular signaling, proliferation and apoptosis. In this trial, patients were to receive infusions of the anti-PKC oligonucleotide under a typical dose-escalation scheme. Having learned from the experience with the Bryostatin-1 trial, we decided to try and examine the effects of the drug on the tumor tissue itself and so we obtained approval from the IRB to obtain, with appropriate informed consent, biopsies of the tumors tissue before and during therapy. These biopsies would then be fixed and paraffin embedded for subsequent immunohistochemical analysis. Samples would be stained for PKC-alpha levels, the presence of the drug, the level of proliferation by staining for the marker Ki67 and for the presence of apoptotic cells.

In addition, peripheral blood samples would be collected and the granulocytes and lymphocytes isolated and RNA prepared from them. The level of PKC-alpha mRNA would then be measured by another lab to determine the mRNA levels were suppressed.

Anti-sense clinical trial.

In all 16 patients were treated with different concentrations of the oligonucleotide and samples were obtained and processed as described below.

Patient blood was drawn by into heparinized tubes and then processed using Polymorphprep (Nycomed, Oslo Norway) to isolate peripheral lymphocytes and granulocytes following the manufacturer's instructions. Briefly, undiluted blood (5 ml) was layered onto 3.5 ml of Polymorphprep in a 15 ml centrifuge tube. Two to three tubes were processed per patient depending on the volume of blood collected. The tubes were spun at 450 x g for 35 minutes in a swing-out rotor at 20°C. This resulted in the pelleting of the red blood cells and the formation of two nucleated cell bands within the separation medium. After aspiration of the plasma, the upper band containing the lymphocytes was removed to a separate tube, and subsequently the lower band containing the granulocytes was removed and placed in another tube. Sterile phosphate buffered saline (PBS, 6-10 ml) was then added to the tubes containing the isolated cells which were then inverted to mix them and spun at approximately 800 x g to pellet the cells. The resultant washed cell pellets were then re-suspended in RNA lysis solution and stored frozen at -80°C until analyzed.

Processing Patient Biopsies:

Biopsies were either immediately frozen on dry ice and then stored at -80°C until analyzed or fixed in 10% neutral buffered formalin for 1 hour and then imbedded in paraffin. A section of each block was stained with H&E using standard methods to determine the nature of the tissue sampled.

Immunohistochemistry:

Sections (five microns) were cut from the paraffin blocks containing the patient biopsies and stained to look at various markers.

KI67 staining.

Actively proliferating cells were identified by staining for the proliferation associated nuclear antigen KI-67 using an antibody from Novocastra Laboratories Ltd. (NCL-Ki67-MM1, Newcastle-upon-Tyne, UK). The slides were deparaffinized, re-hydrated to distilled water and heated in a microwave pressure cooker for 30 minutes in 0.01 M citrate buffer pH 6 to unmask the antigen. They were then washed in PBS, treated with 1.5% hydrogen peroxide in methanol for 10 minutes to inactivate endogenous peroxidases and washed with distilled water. After incubation with blocking solution (2% normal horse serum, 5% BSA in PBS) for 30 minutes the slides were incubated with the anti-KI-67 monoclonal antibody diluted 1:200 in blocking solution for 1 hour. After washing in PBS (2 x 5 minutes) the areas of immunoreaction were detected using Vectastain Elite ABC reagent (Vector Labs, Burlingame, CA) and diaminobenzidine (DAB). The sections were counterstained lightly with hematoxylin and cover-slipped.

Drug staining:

Areas containing the anti-sense oligonucleotide were stained using a monoclonal antibody against the drug. Samples were deparaffinized re-hydrated and the endogenous peroxidases inhibited by treatment with 0.3% hydrogen peroxide. After digestion with 20 micrograms per ml proteinase K (Boehringer) for 15 minutes to retrieve the antigen, the sections were blocked with normal donkey serum (Jackson Laboratories) for 30 minutes, and then stained with a 1:200 dilution of the anti-oligonucleotide monoclonal antibody for 1 hour. The sections were then washed and areas of immunoreaction detected with HRP conjugated donkey anti-mouse antibodies and DAB.

Apoptosis detection:

The level of apoptosis in the biopsy sections was determined by TUNEL assay using TACS system from Trevigen (Gaithersburg, MD) following the directions provided. Briefly, the sections are deparaffinized, re-hydrated with PBS and then treated with proteinase K for 10 minutes. After quenching endogenous peroxidases by treatment with hydrogen peroxide (30% in water), DNA strand-breaks are labeled with biotin labeled nucleotides by the action of terminal deoxynucleotide transferase. Areas of labeling are detected by incubation with horseradish peroxidase labeled streptavidin followed by reaction with DAB. The slides are then lightly counter-stained with methyl green and cover-slipped. Cells undergoing apoptosis can be recognized by their brown-staining nuclei that show the characteristic apoptotic morphology.

PKC-alpha staining

Sections were stained for PKC-alpha using a rabbit anti-PKC-alpha antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Samples were deparaffinized re-hydrated and the endogenous peroxidases inhibited by treatment with 0.3% hydrogen peroxide. After digestion with 20 micrograms per ml proteinase K (Boehringer) for 5 minutes to retrieve the antigen, the sections were blocked with normal donkey serum (Jackson Laboratories) for 30 minutes, and then stained with 1 ug/ml anti-PKC-alpha for 1 hour. The sections were then washed and areas of immunoreaction detected with HRP conjugated donkey anti-rabbit antibodies and DAB.

At this point, all of the tissues and other samples for the trial have been collected and we are in the process of staining any analyzing the materials. We have some preliminary data that is quite encouraging, which suggests that we can detect reasonable levels of the drug within the samples and that the staining for the other markers is working well. We will not, however, complete the analysis until all of the data are in because all sections are being processed in a blinded fashion to try and eliminate unintentional operator bias. We anticipate that the processing and analysis of the study will be complete shortly and when the study is published the support from the USAMRDC will, of course, be gratefully acknowledged.

We anticipate that areas of the tumor that have taken up significant amounts of the anti-sense oligonucleotide will contain less PKC-alpha. In these areas we would also expect to see that fewer cells stain with the proliferation marker Ki67 and that more TUNEL positive cells may be found indicating that the treatment has increased the level of apoptosis in the section.

Conclusions

In this Final Report for the grant DAMD17-94-J-4166 we describe the work that we have done over the 4 years of support. During the course of the project, the direction of the studies have changed somewhat and it is an extremely valuable feature of the mechanism that this flexibility is possible. In this section we will summarize our most important findings and describe where future studies should and will be directed. Our initial experiments to characterize the pattern of PKC isoform expression in a panel of human breast cancer cell lines and to correlate this pattern with the invasive and metastatic potential of the cells was quite successful. The expression of 3 isoforms could be linked in a convincing way with the behavior of the cells. The effect of the overexpression of PKC-alpha in a normally poorly invasive cell line was predicted by our study and we believe that a good immunohistochemical study of PKC-alpha levels in the primary tumor as a marker of outcome should be conducted. Similarly, the interesting finding that more aggressive cells have less PKC-eta should be investigated further. One would predict that overexpression of PKC-eta in an aggressive breast cancer cell line might result in decreased aggressive behavior. Conversely, ablation of PKC-eta in a less invasive cell line might push that cell towards a more aggressive phenotype. This could be investigated with the use of an anti-sense strategy or with the use of ribozymes. It would suggest that an anti-PKC-eta targeted drug might be a poor choice.

Having shifted the emphasis of the project to have a more clinical emphasis, some of the data that we have generated that is the hardest to interpret is in fact quite valuable. The variation that is seen in the levels of MMP-9 in the plasma of patients over relatively short periods of time is quite dramatic. These samples were collected and processed with great care to ensure that random variation was not introduced needlessly. That we achieved this goal is demonstrated by the singular lack of variation in the MMP-2 levels seen in the same samples. We are at a loss to explain these variations, but it makes clear that the levels of this protein is under highly dynamic regulation in the plasma. What role it may be playing is unclear, and it is also unclear if this is the result of the disease process. In the past, investigators have proposed that the level of MMP-9 in patient plasma be used as a marker for tumor load. This clearly is called into question by our data. We are in the process of obtaining IRB approval for a study of MMP levels in the plasma of normal volunteers over time in an attempt to answer these questions.

The variation and difficulty in the interpretation of the PKC data generated was quite disappointing, however, the results were quite informative and have helped us refine our study design for future ongoing projects. Also, we hope that with the additional insight that knowing the level of drug that was achieved in the patient will give us, we will be able to glean additional information from these data. These problems aside, we did make an important finding: the consistent suppression of PKC eta that is produced by treatment with Bryostatin-1. This result is perhaps the more impressive since it was seen against the background of such variation. It is interesting to note that in the first part of our studies, low PKC-eta levels were associated with more aggressive behavior. Thus, one might suppose that suppression of PKC-eta levels might be deleterious. We hope that our new continuous infusion Bryostatin-1 trial in which we will be obtaining tumor samples before and during therapy will allow us to see if these tissues behave the same way as PBLs in the face of Bryostatin-1.

We are awaiting with great anticipation the completion of the anti-sense trial. This trial will be one of the first in which such a detailed study of the disposition, action and effect of a drug has been conducted in tumor tissues and we expect it will provide extremely interesting data.

In closing we would like to take this opportunity to thank the USAMRDC for the support that they have provided through this Career Development Award. The support has provided the freedom from routine duties that has allowed a considerable number of fruitful collaborations and the ability to engage in some exciting correlative science studies.

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List of abbreviations used:

BSA	Bovine Serum Albumin
CAT	Chloramphenicol Acyl Transferase
cd	Clusters of Differentiation
°C	Celsius
cm	Centimeters
DAB	Diaminobenzidine
DT	Developmental Therapeutics
ECL	Enhanced Chemi-Luminescence
EC50	50% Effective concentration
EDTA	Ethylene Diamine Tetra Acetic Acid
FCS	Fetal Calf Serum
HRP	Horseradish Peroxidase
IMEM	Improved Modified Eagles Medium
IRB	Institutional Review Board
K	Thousand
KDa	KiloDaltons
MAPK	Mitogen Activated Protein Kinase
MCF	Michigan Cancer Foundation
µg	Microgram
ml	Milliliter
mM	Millimolar
M	Molar
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger Ribonucleic Acid
NFDM	Non-Fat Dried Milk
nM	Nanomolar
PAGE	Polly-Acrylamide Gel Electrophoresis
PBLs	Peripheral Blood Lymphocytes
PBS	Phosphate Buffered Saline
PBST	PBS with 0.1% Tween-20
PKC	Protein Kinase C
RPM	Revolutions Per Minute
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
Sq	Square
TPA	12-O-tetradecanoylphorbol-13-acetate

List of persons paid from DAMD 17-94-4166

Michael D. Johnson