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13. ABSTRACT (Maximum 200 Words)

We showed that breast cancer MCF7 cells posses a distinct regulation of proteolysis executed by the multicorn when compared with non-cancerous MCF10A cells. The apparent lower total activity of the multicorn in MCF7 cells may constitute an important link between the overall efficiency of cell division and nuclear and cytosolic proteolysis. Regulation of the assembly of the large and small forms of multicorn is accomplished through phosphorylation of their subunits. On the basis of our data we suspect that multicorn constitutes an important player in cellular protein turnover and in regulation of cell cycle. Its distinct properties in the control and cancerous cells strongly suggest that the multicorn may represent an attractive drug target and a marker of physiological state of the cells. Since it has been suspected that both proteasome and the multicorn may share some of their functions, we tested the performance of cancerous and control cells treated with inhibitors of the proteasome, the multicorn, and both the inhibitors combined. We found that high doses of multicorn or proteasome inhibitors are toxic to the cells. However, we determined that a combination of low doses of both inhibitors effectively kills the cancerous cells allowing the noncancerous cells to recover.

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INTRODUCTION

Proper regulation of cell division and cell differentiation are the major factors preventing the neoplastic growth. Proteolysis is one of the widely accepted controlling mechanisms of these processes. The giant multifunctional enzyme named the proteasome is responsible for timely removal or activation of critical transcription factors and cell cycle regulatory proteins in the cytosol and nucleus. Inhibition of the proteasomal action leads to apoptosis. The proteasome is an acknowledged anti-cancer drug target and one of specific inhibitors of the proteasome is already approved to treat multiple myeloma and is under extensive clinical trials against other cancers, including breast cancer. We have discovered a new giant proteolytic complex distinct from the proteasome and ubiquitous among Eukaryotes (Osmulski and Gaczynska, 1998). The enzyme named multicorn apparently takes part in the cell cycle regulation and is involved in partial overcoming the physiological effects of proteasome inhibitors (Glass et al., 1998). Up to date, we found significant differences in the activity, amount, oligomerization status, posttranslational modifications and subcellular localization of the multicorn in human breast cancer MCF-7 cells, as compared with non-cancerous MCF-10A cells. Notably, the total activity of the multicorn seems to be several-fold lower in cancerous cells than in control cells, and this result was confirmed with several methods, both in vitro and in living cells. With the help of unique fluorogenic model substrate especially designed and synthesized in our laboratory for *in vivo* studies, we observed that the proteolytically active multicorn congregates in the nuclear envelope on the onset of mitosis in the control noncancerous cells. In the carcinoma MCF7 cells, however, the nuclear envelope localization was not limited to the onset of mitosis and was already prominent in G2 phase. Since the multicorn emerges as a second giant protease after the proteasome to take part in cell cycle regulation, we decided to test the combined influence of proteasomal inhibitors and inhibitors of the multicorn activity on the survival of cultured cancerous and noncancerous cells. The initial results of experiments now in progress are very encouraging. We observed that cancerous cells are much more vulnerable than the control cells to cell death caused by the combined treatment with low doses of the inhibitors of the multicorn and proteasome activities. Our findings confirm that the multicorn can be a useful anti-cancer drug target and a marker of neoplastic transformation. We will continue our studies on the best ways to utilize the multicorn to specifically target breast cancer cells.

BODY

Up to date, the research accomplishments associated with the objectives and tasks outlined in the approved Statement of Work are as follows:

Objective 1. Cloning and expressing the gene of human multicorn monomer.

***Task 1**: months 1 - 12; molecular basis of different physical and chemical properties of the two multicorn subunits will be studied using a combination of peptide mapping, sequencing and mass spectroscopy.*

Most of the task has been completed We established that the human multicorn is built from a single 150 kDa subunit, similarly to the previously described by our group multicorn from fission yeast (*Schizosaccharomyces pombe*; Osmulski and Gaczynska, 1998). The 150 kDa subunit can be phosphorylated on several distinct serine residues to render polypeptides of electrophoretic mobilities of 165 kDa, 170 kDa and 240 kDa.

In cooperation with the UTHSCSA Mass Spectroscopy core facility, we plan to finalize study on a phosphorylation pattern of the multicorn this summer. The facility recently acquired a new MALDI-TOF CID (collision induced dissociation) spectrometer (Voyager-DE STR Biospectrometry Workstation) enabling relatively a straightforward detection and identification of multiple modifications of peptides. The instrument was set up late March this year and recently passed tests for the quality assurance. We hope that the instrument will also be valuable in the additional efforts of multicorn sequencing. The pure multicorn protein in mg quantities of the large, small, and monomeric forms were prepared to finalize this task.

Task 2: *months 1 - 6; cloning the gene, or genes, encoding the human multicorn monomers using HeLa cDNA library and PCR technology. It will be determined if the two types of subunits are encoded by the same gene.*

As reported before, we established that the 150 kDa and 170 kDa subunits have to be encoded by the same gene, since the 170 kDa polypeptide, and other multicorn polypeptides discovered as a part of this project, are posttranslationally modified forms of the 150 kDa protein.

Our prolonged efforts to clone a full size active multicorn have not been successful so far. We tested several leads resulting from BLAST (NCBI) searches for possible human homologs of the multicorn. A KIAA1716 protein proposed previously was identified as centuarin beta 5. Indeed, it does contain a plecstrin homology domain (PH) as the *S. pombe* homolog would suggest, however its GTPase activity for ADP ribosylation excludes it as a serious candidate. Similarly, a shorter homolog of centuarin, a DDEF-1 protein (KOG0S21), is also presumed a GTPase rather than protease and will not be pursued any longer as a major candidate.
The possible lead derived from a RP11-87M1 clone on a human chromosome 9 has not been followed yet. We plan to pursue this lead this summer. Furthermore, we will closely scrutinize the Sip3p lead. After additional BLAST searches we found that one of the closest matches in human genome sequence, a Sip3p protein, points at cullin 7 and a family of the cullin-like proteins. All of them exhibit a high molecular weight of their respective monomers (a 150-180kDa range) corresponding to amino acid sequences 1500-1700 residue long. They contain several protein binding domains and participate in a formation of high molecular weight complexes, most notably an SCF E3 ligase complex being a part of the proteasome-Ub pathway. Probably, the most tempting target is a protein of unknown function coded by a sequence O75188 consisting of 1753 residues as the closest sequential match of the multicorn homolog.
At the same time, we plan to molecularly disrupt expression of a TPP II peptidase in a mcf10A cell line. This procedure will facilitate a distinction between these two proteins,

which tend to cross-contaminate each other in all the steps of their purification and effectively confusing this way many sequencing efforts. A similar approach with the multicorn gene in *S. pombe* helped to establish the presence of a single major protease of the high molecular weight capable to digest the unblocked AAF and AFK peptide substrates.
There is a chance that there is no homolog of the *S. pombe* multicorn in humans, but only a functional analog. Additional aggressive sequencing efforts executed on the TPPII deletion mutant cells will allow us to finally solve this problem.

Task 3: *months 6 - 18; expressing the gene(s) in Schizosaccharomyces pombe or mammalian expression system. The goal of this task is to obtain active recombinant multicorn molecules characterized by controlled content of the specific monomers.*

The work on this task will begin as soon as the cloned gene encoding the multicorn subunit will be available.

Objective 2. Studying the mechanism controlling the multicorn activity through its oligomerization and phosphorylation.

Task 1: *months 6 - 24; The multicorn complexes of different supramolecular organization and posttranslational modifications will be separated and purified. The qualitative and quantitative parameters of proteolysis catalyzed by these distinct complexes will be determined using selected protein substrates and model fluorogenic peptide substrates.*

The essential part of the task has been completed, as described in previous Reports. We found that the large form of the multicorn (about 4,000 kDa) is built mostly from the 170 kDa and small amounts of the 165 kDa and 240 kDa phosphorylated forms of the 150 kDa multicorn subunit. We found that the small form of the multicorn (900 kDa) is assembled mostly from the phosphorylated 240 kDa and non-phosphorylated 150 kDa polypeptides, and trace amounts of the phosphorylated 170 kDa protein. We identified the 170 kDa form as the one responsible for most, if not all of the proteolytic activity detected with the model peptide substrate, AlaAlaPhe-methylcoumarin (AAF-MCA). To determine if the 170 kDa form is the only form with a functional active site and to identify the actual active center we decided to perform radioactive labeling of the center followed by sequencing. Up to date, we completed the first part of this project, the synthesis of radioactively labeled specific inhibitor of the multicorn, [H^3]AAF-CMK (AlaAlaPhe-chloromethylketone). Planned identification of the active site is especially important for the future design of high-affinity specific inhibitors of the multicorn. Such inhibitors may become useful drugs in the light of our results described below (Task 1 of Objective 3).

The outline of synthesis of H-[^{3}H]Ala-Ala-Phe-chloromethylketone (-CMK)
Boc-[^{3}H]Ala-OH:

t-Butyloxycarbonyl (Boc) protecting group was coupled to the N-terminus of the amino acid using the standard procedure described in the literature (Tarbell et al., 1971).
H-Ala-OH and H-[^{3}H]Ala-OH (0.5 mCi) in a mixture of 1,4-dioxane, water and 1 M NaOH were stirred and cooled in an ice/water bath. Di-tert-butylpyrrocarbonate was added in portions, pH adjusted to 8-9, and stirring continued overnight in the room temperature. The solution was concentrated *in vacuo*, cooled in an ice/water bath, covered with a layer of ethyl acetate and acidified with 1 M potassium hydrogensulfate to pH 2-3. The aqueous phase was extracted with ethyl acetate. The organic extracts were pooled, washed with water, dried over anhydrous magnesium sulfate and crystallized from ethyl acetate/hexane.
Boc-Ala-Phe-benzyl ester (-OBzl): To Boc-Ala-OH and H-Phe-OBzl dissolved in dimethylformamide 1-hydroxybenzotriazole was added and the solution was cooled to 0°C. O-Benzotriazole-N,N,N'N'-tetramethyluronium-hexafluorophosphate (HBTU) was added followed by triethylamine and the reaction mixture was left stirring overnight.
The solvent was removed *in vacuo* and the oil residue dissolved in ethyl acetate and washed with 1 M potassium hydrogensulfate, 5% sodium hydrogencarbonate and saturated sodium chloride. Organic layer was dried over anhydrous magnesium sulfate. Crystallization from ethyl acetate/hexane gave pure product.
TFA x H-Ala-Phe-OBzl: Boc group was removed using 50%TFA in dichloromethane solution. The product was precipitated with diethyl ether.
Boc-[^{3}H]Ala-Ala-Phe-OBzl: Coupling of Boc-[^{3}H]-Ala-OH to H-Ala-Phe-OBzl was carried out as above (see: Boc-Ala-Phe-OBzl).
Boc-[^{3}H]Ala-Ala-Phe-OH: To an argon purged solution of Boc-[^{3}H]Ala-Ala-Phe-OBzl in tetrahydrofuran catalytic amounts of palladium on activated carbon (Pd/C, 10%) was added. Hydrogen gas was passed through the solution until thin layer chromatography (TLC) revealed the completeness of benzyl ester removal. The solution was again purged with argon and filtered. The solvent was evaporated.
Diazomethane production: Diazald was dissolved in anhydrous diethyl ether, cooled and mixed with the cooled solution of KOH in ethanol. The mixture stayed in an ice-bath for 15 minutes, and then diazomethane was distilled cautiously to the receiving flask cooled in an ice-bath and containing small amount of anhydrous diethyl ether.
Boc-[^{3}H]Ala-Ala-Phe-diazomethylketone: Boc-AAF-OH was dissolved in anhydrous THF and cooled to -15°C (ice/ethanol). N-methylmorpholine was added followed by isobutyl chloroformate. The mixture was allowed to react in -15°C for 15 min. to form a mixed anhydride.
The solution of diazomethane in diethyl ether was added and the reaction mixture left stirring for 4 hours. The temperature was allowed to gradually increase to 0°C. Then the reaction mixture was placed to a cold cabinet for several hours.
The remaining diazomethane and the solvent were removed *in vacuo*.
HCl x H-[^{3}H]Ala-Ala-Phe-CMK: Boc-[^{3}H]Ala-Ala-Phe-diazomethylketone was dissolved in anhydrous diethyl ether. The solution was cooled in an ice-bath and gas hydrochloride was bubbled through for 1 hour. The reaction mixture was kept in a cold cabinet for additional 24h.
The white precipitate was filtered off, dissolved in water and lyophilized.
The crude product was purified using reverse-phase high performance liquid chromatography (RP-HPLC; XTerra Prep MS C_{18} column, 5 μm, 19 x 50 mm). Characteristics of pure product: R_T = 25.59 min (RP-HPLC; XTerra RP C_{18}, 5 μm, 4.6 x

250 mm column, gradient 100%A to 100% B, 60 min., where A: water + 0.1% trifluoroacetic acid (TFA), B: 80% acetonitrile/water + 0.1% TFA), MS: 339.90 Da, calc. 339.91 Da.
Purified large form of the multicorn will be treated with the inhibitor, run on SDS-PAGE, and the multicorn subunit will be subjected to in-gel digestion with trypsin. The radioactively labeled tryptic fragment will be sequenced.

Objective 3. Molecular characterization of the multicorn at different stages of the cell cycle.

***Task 1**: months 12 - 18; we will perform flow cytometric analysis of nonsynchronous MCF-7 and MCF-10A cells stained with anti-multicorn antibodies and with propidium iodide (DNA).*

The essential part of experiments has been completed and the data are now under analysis. To support the flow cytometry data, we performed fluorescence microscopy experiments with model fluorogenic peptide substrate AAF-CMAC (AlaAlaPhe-7-amino-4-chloromethylcoumarin), described in previous Report and below.
The multicorn shares many common features with the proteasome, which is an acknowledged anticancer drug target and a major intracellular protease. The functional coupling of the proteasome and the multicorn has long been suspected (Glas et al., 1998). Therefore, we decided to supplement the characterization of the role of multicorn by studies of cells treated by the inhibitor of the multicorn, by the inhibitor of the proteasome, and by a combination of both inhibitors. Predictably, high doses of multicorn or proteasome inhibitors were toxic to the cells. However, we determined that a combination of low doses of both inhibitors effectively killed the cancerous cells allowing the noncancerous cells to recover.

Determination of survival curves for MCF7 and MCF10A cells treated with the multicorn and the proteasome inhibitors.
Experimental design: We choose AAF-CMK (AlaAlaPhe-chloromethylketone) and a peptide boronic acid derivative MG262 (carbobenzoxy-LeuLeuLeu-$B(OH)_2$; proteasome inhibitor III) as inhibitors of the multicorn and the proteasome, respectively (available from Sigma and Calbiochem). AAF-CMK competitively restrains the proteolytic activity of the multicorn (Osmulski and Gaczynska, 1998) and tripeptidyl peptidase II (TPPII; Geier et al., 1999). In our biochemical studies with MCF cell extracts the vast majority of the activity affected by AAF-CMK belonged to the multicorn and only traces to the TPPII. Even if part of the observed effects stems from inhibition of both the multicorn and the TPPII, this does not diminish the practical value of our studies on the usefulness of treatment of cells with multiple inhibitors. The usefulness of AAF-CMK in cell culture studies is well documented (Glas et al., 1998). MG262 on the other hand is a highly specific, high-affinity inhibitor of the proteasome (Gaczynska and Osmulski, 2002). It is a close homologue of the drug bortezomib (PS341, Velcade) with the same mechanism of action against the proteasome as the drug approved to treat multiple myeloma and tested against other cancers (Adams, J., 2002).

First, we tested an array of the following conditions of treatment of the MCF7 and MCF10A cells in culture:

(1) Control: 1% (volume) of the solvent used for the inhibitors (DMSO; dimethylsulfoxide) added to the culture medium;
(2)-(6) Treatment with AAF-CMK: 1 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM;
(7)-(10) Treatment with MG262: 10 nM, 20 nM, 50 nM, 100 nM;
(11)-(14) Treatment with the mixtures of 5 μM AAF-CMK and 10 nM, 20 nM, 50 nM or 100 nM MG262;
(15)-(18) Treatment with the mixtures of 10 μM AAF-CMK and 10 nM, 20 nM, 50 nM or 100 nM MG262;
(18)-(21) Treatment with the mixtures of 20 μM AAF-CMK and 10 nM, 20 nM, 50 nM or 100 nM MG262.

The concentrations of single inhibitors were choosen based on the already known in vitro and in vivo data (Glas et al., 1998; Gaczynska and Osmulski, 2002). The cells were cultured in 24 - well plates. After 12, 24 and 48 hours of culture the cells were counted with trypan blue to determine total cell number and the number of dead cells.

The aim of this experiment was two-fold. Firstly, we wanted to test if any of the mixtures of inhibitors will show synergistic effects on the cell survival. Secondly, we hoped to detect differences in the response of cancerous and noncancerous cells to the treatments.

Results.

1. The AAF-CMK inhibitor in high concentrations was toxic to both MCF7 and MCF10A cells. After 48 hours of exposure to 100 μM AAF-CMK, more than 70% of both MCF7 and MCF10A cells stained with trypan blue (were dead), and the count of live cells were less than 10% of the control count. For comparison, less than 1% of total cell number in control populations stained with trypan blue. Lower concentrations of AAF-CMK exhibited moderate effect on survival of the cells and significant effect on their proliferation. For example, after treatment with 10 μM AAF-CMK live cell count reached 40-50% of the control count and dead cells comprised only up to 10% of the total cells counted. No systematic differences in proliferation or survival were observed between MCF7 and MCF10A cells.

2. We confirmed the notion that cancerous cells are more vulnerable to the actions of the proteasome inhibitor than the noncancerous cells (Adams, 2002). As shown in Figure 1, the percent of the dead cancerous MCF7 cells systematically increased with increasing concentrations of MG262 inhibitor, reaching more than 60% after 48 hours of exposure to the compound. The percent of dead noncancerous cells never reached 20 under the same conditions. The count of live MCF7 cells after treatment with the highest used concentration of MG262 (100 nM) reached only about 10% of the control cell count. The noncancerous MCF10A cells reached 50% of the control live cell count under the same conditions.

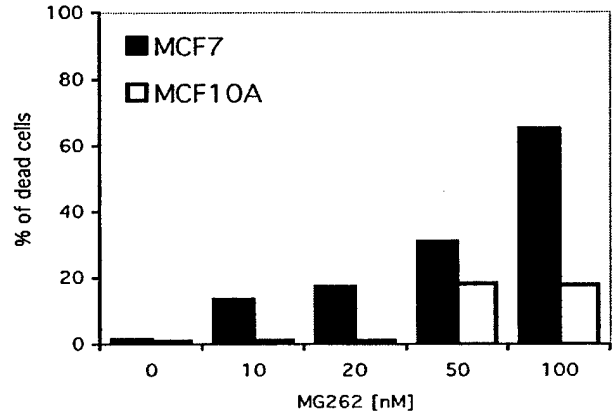

Figure 1. The breast cancer cells MCF7 are more susceptible to the actions of the proteasome inhibitor MG262 than noncancerous breast cells MCF10A. The cells were treated with indicated concentrations of the inhibitor for 48 hours. Percent of dead cells was calculated after counting cells stained with trypan blue. The bars represent averages from at least three counted fields.

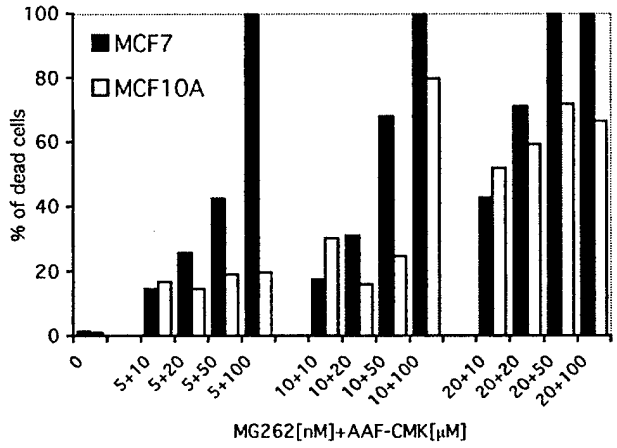

Figure 2. Treatment of cultured cells with the multicorn and proteasome inhibitors in mixture is more deadly for the breast cancer MCF7 cells than for the noncancerous MCF10A cells. The cells were treated for 48 hours and counted as in Figure 1. It is worth to notice that at least some of the mixtures leave wider "window" between responses of the MCF7 and MCF10A cells than the MG262 inhibitor alone (Figure 1).

3. When cells in culture were treated with the two inhibitors combined, the effects were deadly especially for the MCF7 cells with higher concentrations of the inhibitors (Figure 2). The noncancerous MCF10A cells were significantly less prone to die out when treated with the inhibitors. In order to learn which of the conditions show the highest synergistic effect of the two inhibitors, we calculated the ratios of the dead cell index (% of dead cells) for the mixture of inhibitors to the geometric average of the sum of dead cell indexes for the appropriate single-inhibitor treatments. The high ratios indicated strong synergistic effects of the treatment with the compounds in mixture. The ratios were highest for MCF7 cells for the following mixtures of inhibitors: 10 μM + 50 nM (AAF-CMK + MG262), 10 μM + 100 nM, 20 μM + 20 nM and 20 μM + 50 nM. To select the best conditions differentiating between MCF7 and MCF10A cells, we divided the appropriate ratios for MCF7 cells by the ratios calculated for MCF10A cells. The high "MCF7/MCF10A index" indicated that the particular conditions were significantly more deadly for the cancerous cells than the control cells. Similar set of calculations was performed with the total cell count instead of dead cell index. In this case, of course, lowest calculated ratios were the most favorable to differentiate between MCF7 and MCF10A cells. Both sets of calculations returned very similar results, with 10 μM + 50 nM (AAF-CMK + MG262) and 20 μM + 20 nM mixtures offering the widest window between cancerous and control cells.

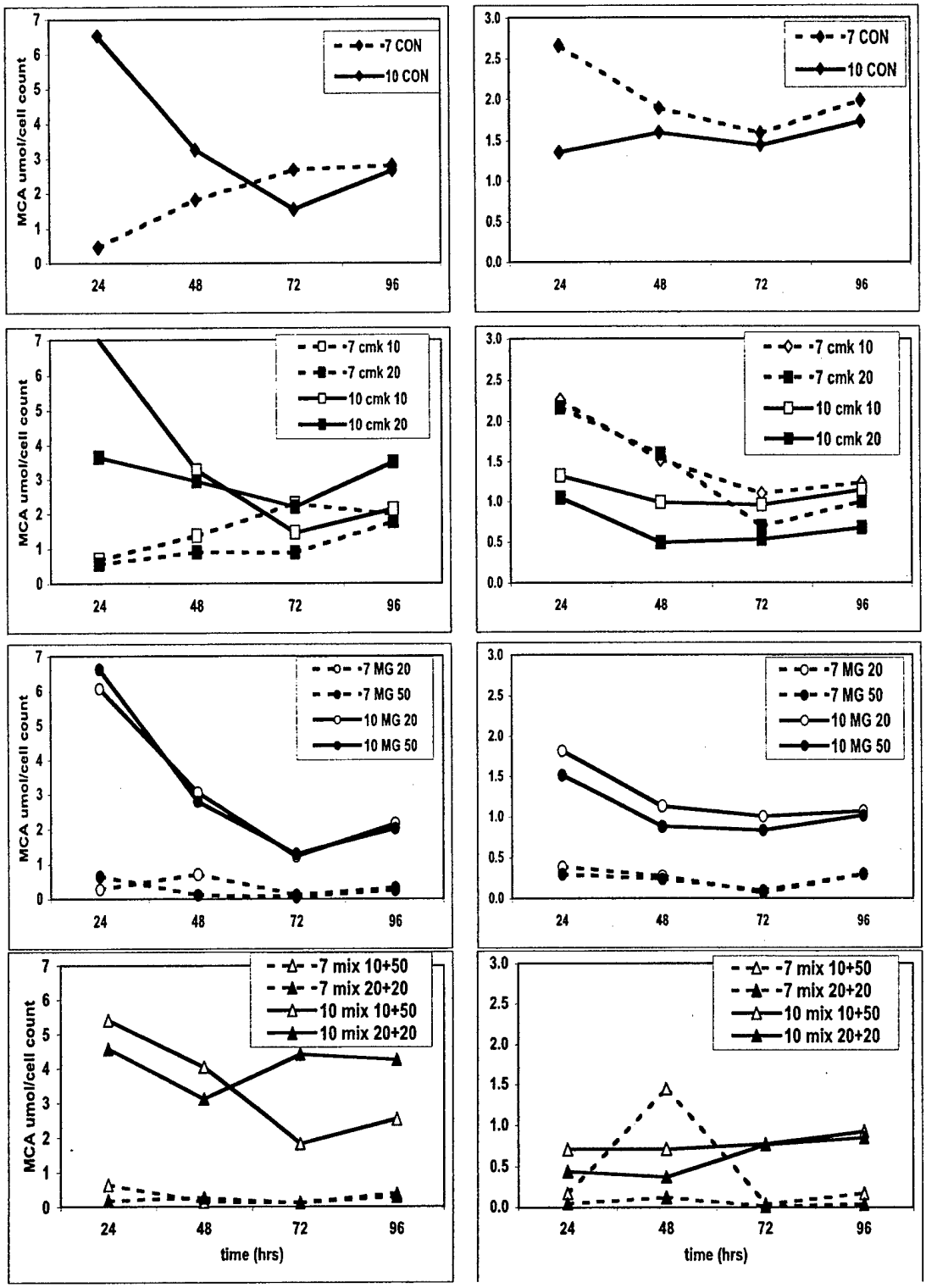

Figure 3. Peptidase activity of multicorn (left panels) and proteasome (right panels) were tested *in vivo* in the MCF7 and MCF10A cultured cell lines using AAF-MCA and SucLLVY-MCA substrates, respectively. See text for descriptions.

Determination of survival curves and activities of the multicorn and the proteasome in cells treated with the most promising combinations of the two inhibitors.

Experimental design: we choose the best mixtures of the two inhibitors, as determined in the previous experiment, to characterize in depth the response of MCF7 and MCF10A cells to the treatments. As before, the cells were cultured in 24-well plates and treated with the solvent alone, the single inhibitors (10 μM AAF-CMK, 20 μM AAF-CMK, 20 nM MG262 and 50 nM MG262) and the mixtures of 10 μM + 50 nM (AAF-CMK + MG262) and 20 μM + 20 nM. The following parameters were determined in cells after 24, 48, 72 and 96 hours of the exposure to the inhibitors:

a) cell count and dead cell count (with trypan blue);

b) total content of DNA in lysed cells determined fluorometrically with propidium iodide (PI);

c) total activity of the proteasome determined fluorometrically in lysed cells with the model fluorogenic substrate of the proteasome, succinyl-LeuLeuValTyr-methylcoumarin (SucLLVY-MCA);

d) total activity of the multicorn (and potentially other proteases degrading the substrate used) determined fluorometrically in lysed cells with the model fluorogenic substrate AlaAlaPhe-methylcoumarin (AAF-MCA);

e) activities of caspased tested with fluorogenic substrates: IETD-MCA (caspase 8), DEVD-MCA (caspase 3), and YVAD-MCA (caspase 7).

The aim of this experiment was to characterize in detail the response of cells to combined actions of the two inhibitors.

Results: of the determination of peptidase activities of the multicorn and the proteasome are presented in Figure 3. Other data are processed and will follow soon. So far, we determined that:

Peptidase activity of multicorn (Figure 3, left panels) and proteasome (Figure 3, right panels) were tested *in vivo* in the MCF7 and MCF10A cultured cell lines using AAF-MCA and SucLLVY-MCA substrates, respectively. Panels in a subsequent rows depict activities in control (untreated) cells, treated with AAF-CMK (10 and 20 μM), treated with MG262 (20 and 50 nM), and treated with mixture of the inhibitors (AAF-CMK + MG262). Data for MCF7 cells are represented by broken lines and for MCF10A by solid lines. The cells were treated with specified amount of the inhibitors and the peptidase activities measured at the indicated time intervals directly in 24-well plates where they were cultured.

To make cells permeable for the substrates, the culture medium was removed, the cells were overlaid with 200μL of water and then the cells were exposed to two cycles of freezing at –80°C for 3 hrs. and then thawing them at 37 °C for 30 min. Subsequently, the substrates were added to final concentration 100μM in 20 mM Tris buffer, pH 7. After an incubation at 37°C for 1hr, fluorescence of free MCA released from the substrates as a measure of peptidase activity was measured in the plates using a Kodak ImageStation 2000R with an emission filter set to 445 nm. The fluorescence data were standarized with the known amount of free MCA and expressed as micromoles of released MCA per PI determined cell count.

AAF-MCA activity followed three patters: it was either almost completely inhibited, it increased logarithmically or decayed exponentially. MCF7 cells reacted with almost a complete abolition of the AAF-MCA activity when treated with MG262 or with the mixture of inhibitors. Interestingly, the treatment with AAF-CMK alone did not have a prolonged effect. At the same time, the AAF-MCA activity in MCF10A was decreasing exponentially in all the cases, except the mixtures where it showed constantly about 50% decrease of the activity. Never the complete abolition of the AAF-MCA activity was observed for these cells.

In contrast, proteasome activity expressed as an amount of hydrolyzed SucLLVY-MCA remained relatively constant with time, changing only up to two fold. Surprisingly, MCF10A cells were more sensitive toward the AAF-CMK inhibitor than MCF7 cells. As expected, the cancerous cell line was much more sensitive toward MG262 than the control cells. The treatment with the mixture of inhibitors led to a stronger and faster inhibition of the proteasomal activity than any of the inhibitors acting alone. Only a very limited recovery of the proteasomal activity was observed, especially after treatment with the mixtures.

Taking together, even with such a limited number of tested mixtures of the inhibitors it seems likely that the simultaneous treatment of cells with MG262 and AAF-CMK may induce more pronounced effects faster in the cancerous cells leaving normal cells relatively unaffected. Additionally, the normal cells in contrast with the cancerous ones preserve their capability to at least partially restore the peptidase activities to the original level. The data also stress the importance of a coordinated pharmacological intervention into the intracellular proteolytic system with the special emphasis on the proteasomal pathway.

In concert with this conclusion are our preliminary data on a level of activity of caspases involved in the process of apoptosis. To study activity of these proteases, we used following substrates: IETD-MCA (caspase 8), DEVD-MCA (caspase 3), and YVAD-MCA (caspase 7). A higher level of activity of these enzymes is believed to indicate a higher level of apoptosis. Up to three fold higher level of activity of these caspases was observed in MCF7 than MCF10A cells when the mixtures of the inhibitors were used. This observation may suggest that indeed the cancerous cells respond much stronger to the treatment with the protease inhibitors by turning on the cell death pathway. This line of studies will be vigorously pursued in the near future.

Observation of the activities of the proteasome and the multicorn in living cells.

Experimental design. In the previous Report we described experiments with a multicorn substrate especially designed to perform well *in vivo*. We continued the use of this substrate, AlaAlaPhe-7-amino-4-chloromethylcoumarin (AAF-CMAC) to monitor the activity of the multicorn in living cultured cells. To monitor the activity of the proteasome in a similar manner, we synthesized the fluorogenic substrate succinyl-LeuLeuValTyr-chloromethylcoumarin (SucLLVY-CMAC). Briefly, the CMAC (chloromethylcoumarin) label is characterized by a better retention in living cells than MCA (methylcoumarin) label, without sacrificing a yield of fluorescence and solubility in water-based solvents. The synthesis of SucLLVY-CMAC was performed in a similar manner to the described previously synthesis of AAF-CMAC.

In short, the MCF10A (control) and MCF7 (cancerous) cells were grown on coverslips in a medium containing: (a) 1% solvent (dimethylsulfoxide); (b) 20 μM AAF-CMK (the

multicorn inhibitor); (c) 20 nM MG262 (the proteasome inhibitor) or (d) both inhibitors combined (20 μM AAF-CMK + 20 nM MG262). Coverslips were removed at desired time points from the culture (24 hours, 48 hours etc.), washed with phosphate-buffered saline (PBS), overlayed with 10 μl of PBS containing 500 μM substrates and wet-mounted on microscope slides for analysis under the fluorescent microscope (Zeiss AxioVision 2) with the excitation/emission filter used for DAPI visualization.

The aim of this experiment was to monitor the localization and apparent activity of the multicorn and the proteasome in cells treated with the single inhibitors or their mixtures.

Results

Analyzing data obtained in this experiment is still in progress, and the experiment will be continued with other concentrations of the inhibitors. Up to date, we confirmed that the multicorn and the proteasome respond to the treatment with the inhibitors (see also Figure 3). Upon treatment with the mixture of the inhibitors, we observed dramatic changes in intracellular distribution of both the multicorn and the proteasomal activities in cancerous MCF7 cells. The changes were much less pronounced or absent in the control MCF10A cells (Figures 4 and 5). We are continuing these exciting studies with more time-points and more conditions of treatment with inhibitors.

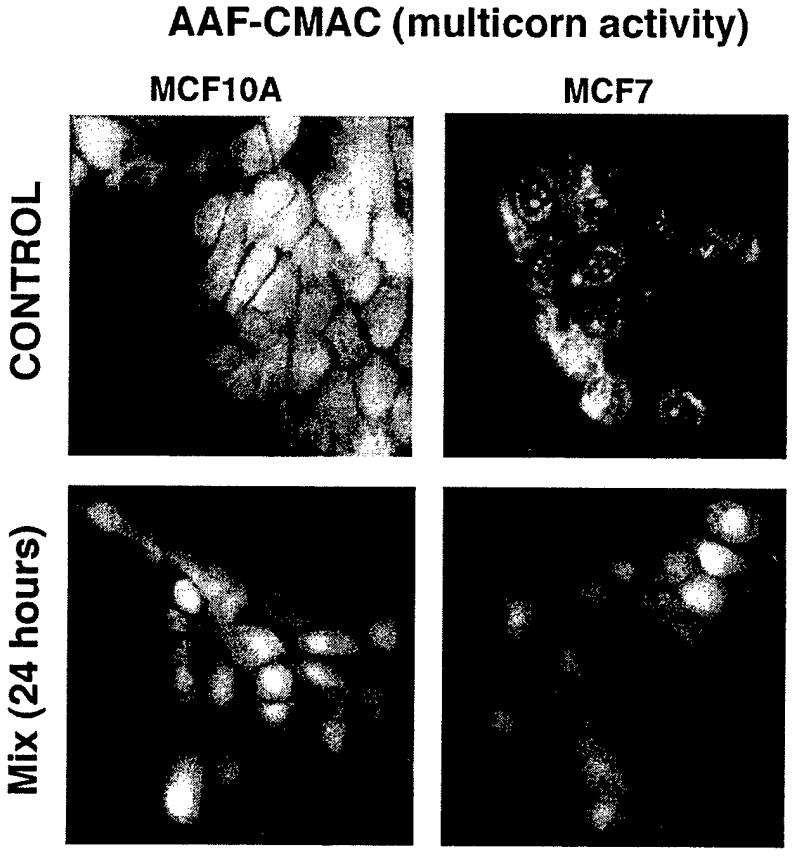

Figure 4. The activity of the multicorn was monitored in living cells with AAF-CMAC fluorogenic substrate. Upper panel: cells were cultured for 24 hours in the presence of 1% (v/v) solvent used to dissolve inhibitors (DMSO). Lower panel: The cells were cultured for 24 hours in the presence of 20 μM AAF-CMK and 20 nM MG262. The intensity of images were brought to similar values for the sake of comparison of intracellular distribution. However, the actual intensity of the fluorescence was up to 30% lower in the cells treated with inhibitors. The change of distribution of the activity from mostly perinuclear and cytosolic (control) to uniformly cytosolic and nuclear (treated) is especially well pronounced in the cancerous MCF7 cells.

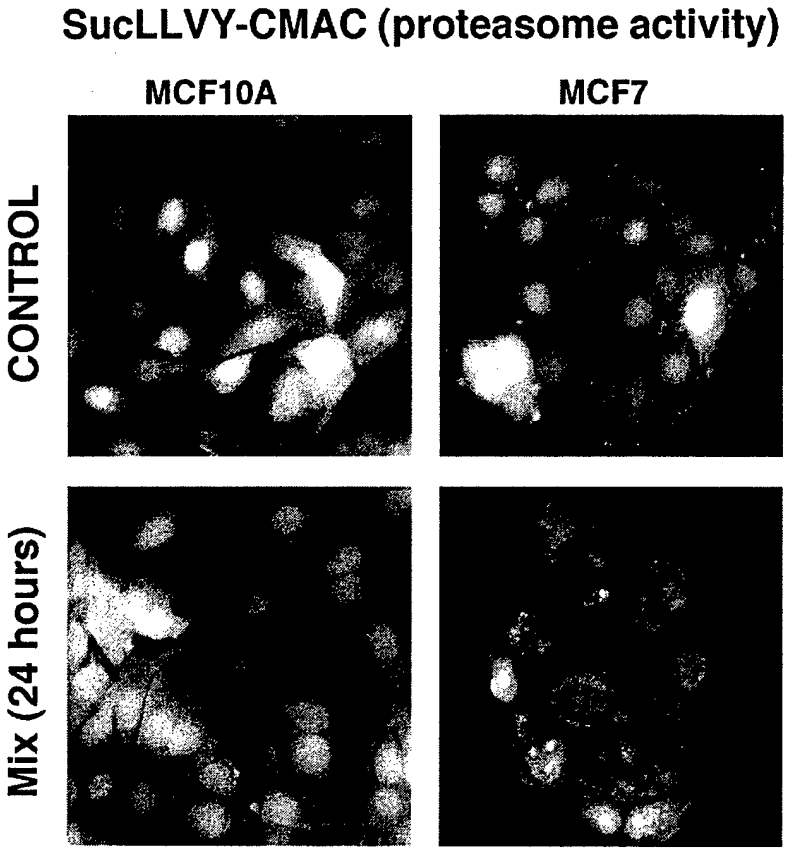

Figure 5. The activity of the multicorn was monitored in living cells with SucLLVY-CMAC fluorogenic substrate. Upper panel: cells were cultured for 24 hours in the presence of 1% (v/v) solvent (DMSO). Lower panel: The cells were cultured for 24 hours in the presence of 20 μM AAF-CMK and 20 nM MG262. The intensity of images were brought to similar values. However, the actual intensity of the fluorescence was about two-fold lower in the treated MCF10A cells and more than six-fold lower in the treated MCF7 cells than in the respective control samples. The change of distribution of the activity from uniformly nuclear and cytosolic (control) to perinuclear and nuclear-focal (treated) is well visible in the cancerous MCF7 cells.

Task 2: months 18 - 36; we will analyze the expression and biochemical properties of the multicorn in synchronized MCF-7 and MCF-10A cells.

The task has been completed and was described in previous Reports. In short, we found that the proteolytically active, phosphorylated large form of the multicorn localized mostly in the cytosol, and also in the nuclear envelope, with cells in G0 exhibiting only a weak cytosolic activity of the protease. The cancerous MCF7 cells exhibited several-fold lower multicorn activity than the control noncancerous MCF10A cells. During mitosis the multicorn activity apparently colocalized with the emerging chromosomes. Interestingly, the dramatic shift of major localization of the multicorn activity from cytosolic to nuclear envelope was a feature of the control cells and not the cancerous cells. In the latter the multicorn activity was detectable both in the cytosol and around nucleus already in G2 phase.

KEY RESEARCH ACCOMPLISHMENTS

(the accomplishments achieved specifically during the recent year are **in bold)**

- We established that the human multicorn is built from a single subunit of apparent molecular mass 150 kDa. We found that the 150 kDa subunit can be phosphorylated on serine residues in several distinct sites to render polypeptides

of electrophoretic mobility 165 kDa, 170 kDa and 240 kDa. Apparently, the 170 kDa form is mostly, if not exclusively, responsible for the observed proteolytic activity of the multicorn complex.

- We discovered that the multicorn, which was first found and described in the cytosol, is detectable with biochemical methods in the nuclear fraction during the onset of mitosis. This observation was later confirmed and clarified by tracking of fluorescent multicorn substrate in living cells.

- We established that the pattern of differently phosphorylated subunits is specific for the particular cellular fraction, and differs between nonsynchronous, mitotic and overconfluent (G0) cells. The pattern in specific subcellular compartments differs between control and cancerous cells.

- We isolated the large and small oligomeric forms of the multicorn and determined their subunit composition in respect to differently phosphorylated polypeptides.

- We found that the large oligomeric form of the multicorn isolated from MCF-10A control cells exhibits several - fold higher specific activity toward a model peptide substrate than the large form isolated from breast cancer cells MCF-7. This difference is accompanied by a distinct pattern of subunit phosphorylation.

- We found that the ratio of large and small forms of the multicorn dramatically differ in cytosols of nonconfluent and overconfluent control MCF-10A cells. The large form was predominant in nonconfluent cells, whereas the small form was the only detectable active form of the multicorn in the cytosol of overconfluent (G0) MCF-10A cells. To the contrary, in cancerous MCF-7 cells the large cytosolic form was always predominant with only a small decline in overconfluent cell culture.

- We synthesized and characterized a unique tool for studies of the multicorn in living cells, a fluorescence probe AAF-CMAC. The probe readily diffused into MCF cells, where was digested with the subsequent release of CMAC. The process did not induce apoptosis. The imaged distribution and intensity of fluorescence reflected the peptidolytic activity of the multicorn in intact cells and was inhibited *in vivo* by an exposure of the cells to AAF-CMAC, the only know inhibitor of the multicorn.

- We found, thet the overall intensity of fluorescence of CMAC in nonsynchronous MCF10A cells is 5 – 10 fold higher than in nonsynchronous MCF7 cells. This result is in perfect agreement with *in vitro* measurements of activities of the multicorn complexes isolated from the two cell lines.

- We observed that the multicorn is unevenly localized in the MCF cells: mostly in cytosol together with membrane structures, but also around/in the nuclear envelope. Moreover, we detected changes in subcellular localization of the multicorn activity during progression of the cell cycle, which followed our

previous observations of the multicorn distribution in *S. pombe*. Specifically, the activity was always present in the cytosol, but only at a very low level in the overconfluent (G0) cells; Foci of strong CMAC fluorescence were detectable in the nucleus when the nuclear membrane was still present, but not in overconfluent (G0) cells; Moreover, a very strong fluorescence around/in the nuclear envelope appeared in late G2 and/or on the onset of mitosis and persisted until the nuclear membrane disappeared. For a brief period during chromatin condensation, a very strong fluorescence co-localized with the emerging chromosomes. Finally, on late stages of the condensation of chromatin, the fluorescence diffused into the cytosol leaving only a faint border around condensed chromosomes.

- The major observed differences between localization and intensity of the CMAC fluorescence in MCF7 and MCF10A cells included the following: (i) the strong fluorescence around nuclear envelope appeared already in G2 in MCF-7 cells, whereas it correlated rather with the onset of mitosis in MCF-10A cells; (ii) the cytoplasmic fluorescence was relatively stronger in MCF10A cells than in MCF7 cells, an observation in concert with biochemical data.

- **We synthesized radioactively labeled specific inhibitor of the multicorn, [H^3]AAF-CMK (tritiated AlaAlaPhe-chloromethylketone) in order to identify the active site of the protease.]**

- **We synthesized the model substrate SucLLVY-CMAC (succinyl-LeuLeuValTyr-7-amino-4-chloromethylcoumarin) especially designed for in vivo studies on the activity of the proteasome, a major protease with apparent functional ties to the multicorn. The substrate is used to monitor the activity of the proteasome in cultured cells treated with the multicorn and proteasome inhibitors.**

- **We tested the survival of cultured MCF7 and MCF10A cells upon treatment with the multicorn inhibitor AAF-CMK, the proteasome inhibitor MG262 and the mixture of both inhibitors. Predictably, high doses of multicorn or proteasome inhibitors were toxic to the cells. However, we determined that a combination of low doses of both inhibitors effectively killed the cancerous cells allowing the noncancerous cells to recover.**

- **We tested the activities of the multicorn and the proteasome in cells treated with single abd combined inhibitors, as above. Surprisingly, MCF10A cells were more sensitive toward the AAF-CMK inhibitor than MCF7 cells. As expected, the cancerous cell line was much more sensitive toward MG262 than the control cells. The treatment with the mixture of inhibitors led to a stronger and faster inhibition of the proteasomal activity than any of the inhibitors acting alone. Only a very limited recovery of the proteasomal activity was observed, especially after treatment with the mixtures.**

- **Upon treatment with the mixture of the multicorn and the proteasome inhibitors, we observed dramatic changes in intracellular distribution of both**

the multicorn and the proteasomal activities in cancerous MCF7 cells. The changes were much less pronounced or absent in the control MCF10A cells.

- **Taking together our initial data obtained with the mixtures of the inhibitors, it seems likely that the simultaneous treatment of cells with MG262 and AAF-CMK may induce more pronounced effects faster in the cancerous cells leaving normal cells relatively unaffected. Additionally, the normal cells in contrast with the cancerous ones preserve their capability to at least partially restore the peptidase activities to the original level. The data also stress the importance of a coordinated pharmacological intervention into the intracellular proteolytic system with the special emphasis on the proteasomal pathway.**

REPORTABLE OUTCOME

1. The radioactively labeled specific inhibitor of the multicorn, [H^3]AAF-CMK (tritiated AlaAlaPhe-chloromethylketone) was synthesized. The inhibitor will be used to identify the active site of the multicorn.

2. The model substrate SucLLVY-CMAC (succinyl-LeuLeuValTyr-7-amino-4-chloromethylcoumarin) especially designed for in vivo studies on the activity of the proteasome, a major protease with apparent functional ties to the multicorn, was synthesized.

3. Parts of the research were included in the invited lecture "Caretaker or Undertaker: The Role of The Proteasome/Ubiquitin System in Aging", which was a part of The Spring 2004 Biology of Aging Seminar Series at The Barshop Center for Longevity and Aging Studies/The San Antonio Nathan Shock Aging Center.

4. Postdoctoral Associate Elzbieta Jankowska was trained in biochemical and cell biology techniques as a part of conducted research. Dr. Jankowska, a chemist, synthesized the AAF-CMAC and SucLLVY-CMAC substrates and the [H^3]AAF-CMK inhibitor, which proved to be an extremely useful tools for multicorn studies.

5. Graduate student in Molecular Medicine, Xiaolin Qin, is trained in a variety of biochemical and cell biology techniques in PI's laboratory.

CONCLUSIONS

We showed that breast cancer MCF7 cells posses a distinct regulation of proteolysis executed by the multicorn when compared with non-cancerous MCF10A cells. The apparent lower total activity of the multicorn in MCF7 cells and only weak changes in subcellular localization may constitute an important link between the overall efficiency of cell division and nuclear and cytosolic proteolysis. Regulation of the assembly of the large and small forms of multicorn is accomplished through a complex phosphorylation pattern of their subunits. Moreover, it seems that the phosphorylation also controls

subcellular localization of the multicorn and ultimately its fate. The cellular distribution of the multicorn, similarly to the best known large intracellular protease, the proteasome, is not limited to cytosol, however the most of the both proteases resides in this compartment. On the basis of our data we suspect that multicorn constitutes an important player in cellular protein turnover and in regulation of cell cycle. Its distinct properties in the control and cancerous cells strongly suggest that the multicorn may represent an attractive drug target and a marker of physiological state of the cells. Since it has been long suspected that both proteasome and the multicorn may share some of their functions (Glas et al., 1998), we tested the performance of cancerous and control cells treated with inhibitors of the proteasome, of the multicorn, and both the inhibitors combined. The results of these tests are very encouraging. High doses of multicorn or proteasome inhibitors are invariably toxic to the cells. However, we determined that a combination of low doses of both inhibitors effectively kills the cancerous cells allowing the noncancerous cells to recover. We are continuing the studies on this very promising subject.

REFERENCES

Adams, J. (2002). Proteasome inhibition: a novel approach to cancer therapy, Trends in Molecular Medicine *8*, S49-54.

Gaczynska, M. O., P. A. (2002). Inhibitor At The Gates, Inhibitor In The Chamber: Allosteric And Competitive Inhibitors Of The Proteasome As Prospective Drugs., Curr Med Chem – Imun, Endoc & Metab Agents *2*, 279-301.

Geier, E., Pfeifer, G., Wilm, M., Lucchiari-Hartz, M., Baumeister, W., Eichmann, K., Niedermann, G. (1999) A giant protease with potential to substitute for some functions of the proteasome. Science *283*, 978-981.

Glas, R., Bogyo, M., McMaster, J. S., Gaczynska, M., and Ploegh, H. L. (1998). A proteolytic system that compensates for loss of proteasome function. Nature *392*, 618-622.

Osmulski, P. A., and Gaczynska, M. (1998). A New Large Proteolytic Complex Distinct From the Proteasome Is Present In the Cytosol Of Fission Yeast. Current Biology *8*, 1023-1026.

Tarbell D.S., Yamamoto Y., Pope B.M. (1971) Proc. Nat. Acad. Sci. 69, 730-732.