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13. Abstract

Histone deacetylase inhibitors (HDAI's) are a promising class of experimental therapeutics. While rapid, butyrate-induced apoptosis is less prominent than among other cancers, cells from 4 of the 6 PrCa lines were irreversibly growth inhibited, especially at low population densities. The JCA-1 cell line continued to replicate in the presence of butyrate or SBHA (another HDAI). The other HDAI-tolerant line, TSU-Pr1 could almost fully recover replication abilities upon removal of butyrate when treated at high cell densities, but was severely inhibited when treated at colony forming assay densities. The mechanisms responsible for these distinct forms of HDAI tolerance are the subject of continuing investigation.

One of the goals of our original proposal was to attempt to establish new cell lines from prostate cancers to achieve a better representation of moderately-well differentiated cancers among available laboratory models. We have been unsuccessful at establishing such lines from prostatectomy-derived tumor tissue, by convention unlikely to represent aggressive disease. Metastatic PrCa cells, obtained by bone marrow biopsy of persons with advanced disease, will be added to dense cultures of normal bone stroma. Initial results with PrCa lines demonstrates feasibility for quantitating colony forming efficiency, including assessment of HDAI sensitivity.

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Introduction

Histone deacetylase inhibitors (HDAI) are a promising new class of anticancer agents (1) the prototypes of which have been butyrate and other short-chain organic acids (SCAs). Over the past three years, nearly a dozen structurally-distinct HDAIs have been discovered, including suberoyl bishydroxamate (SBHA) and several natural products. Because butyrate is both short lived (due to rapid metabolism and excretion) and productive of severe metabolic acidosis (J. McBain, unpublished studies) at the concentrations required for growth inhibition (3-5 mM), the new HDAIs are more feasible for clinical testing. Nonetheless, several experiments of nature (certain organic acidemias and experiences with poisoning by SCA metabolism inhibitors - (2)), and more recent trials of new HDAIs have suggested that this target has virtues for antineoplastic therapy.

Cultured cells treated with butyrate or SBHA almost universally exhibit a cell cycle arrest which is reversible by simple refeeding. A variable proportion of the cell population manifests other changes that depend upon the nature of the cells, such as tissue-typical maturation, expression of differentiation markers/genes, apoptosis or senescence. Because some of these changes follow non-specifically from other means of inhibition of replication in logarithmically growing cells, one of the challenges for investigators of HDAI action is to distinguish those changes from any that might specifically follow the hyperacetylation of nuclear proteins. Our chief aim in this project is to understand how distinct cell lines derived from prostate cancer can exhibit some of these unique responses and long term outcomes despite similarities in growth and differentiation properties in logarithmically-growing cultures. These findings will be relevant to larger issues of normal cell/neoplastic cell distinctions, cancer cell heterogeneity and determinants of cell survival after therapeutic inhibition of growth.

Body

Determinants of recovery from butyrate-induced growth arrest

Our subject cell lines are 6 long-established prostate cancer cell lines – LNCaP (3), ND-1 (4), DU145 (5), PC3(6), TSU-Pr1 (7), and JCA-1 (8). Of this series, only the first is sufficiently well differentiated that it expressed PSA and PSMA, while even the epithelia-specific protein EpCAM is absent from the last 3 cell lines. Because the typical PrCa generally expresses both PSA and EpCAM, the majority of existing PrCa lines appear to represent the minority of PrCa cases - high grade, androgen-independent, PSA-nonexpressing (poorly-differentiated) cases that nonetheless may represent a fully-progressed malignancy (9).

For purposes of mechanistic analysis, the uniformity of cell biological characteristics of the poorly-differentiated PrCa lines provide advantages that compensate for the lack of generalizability. While characteristics like PSA expression are useful measures of PrCa cell differentiation, even butyrate-induced increases in PSA expression in LNCaP cells have been shown to have a large component of androgen-dependency (10). For a similar reason, the extraordinarily serum- and cell density-dependent PC-3 cell line complicates our analysis, especially when the effects of butyrate are so clearly dependent on cell density even in autonomous lines (see below); for this later reason, we have limited our molecular comparisons to the remaining 4 lines. Note however that we are including all six lines as far as presenting the survey of butyrate responsiveness.

For our most recent work on HDAI responsiveness, we have considered ND-1 and DU145 to be typical of butyrate-hypersensitive prostate cancers, and JCA-1 and TSU-Pr1 to be relatively tolerant of butyrate. However, each of the 6 PrCa cell lines is much more sensitive to

butyrate in our hands than are cultures of normal bone stroma, normal fibroblasts, normal prostate epithelia and several cell lines from human colorectal, breast and lung cancers. On the other hand, the high level susceptibility to apoptosis typical of the colorectal cancer cell lines COLO 201 and VACO 5 have NOT been matched by the PrCa lines. Instead, the typical PrCa cell that fails to resume replication following refeeding with butyrate-free medium dies slowly relative to its loss of replication abilities, even if it does eventually exhibit changes typical of apoptosis.

The problems that have presented themselves during our first two years have thus evolved from a prediction that we would find a sharp distinction between sensitivity and resistance to butyrate-induced apoptosis. Since this appears now not to be the case, we have been able to consider butyrate and other HDAIs to be general growth inhibitors, and treated cells to have a range of abilities to recover replication abilities after treatment. Even normal fibroblasts and prostatic epithelial cells exhibit some cell death, especially if treated when in logarithmic growth. We are now impressed with, and have chosen to study two related phenomena associated with the probability of cancer cell recovery from growth arrest. The two cell lines which are the most 'butyrate-tolerant' (JCA-1 and TSU-Pr1) exhibit distinct behaviors depending upon the conditions of the experiment.

A. JCA-1 fails to growth arrest during the first two days of treatment with either butyrate or SBHA. The only similar phenomenon was an early report (11) of the HTC hepatoma cell line that was exposed to gradually increasing butyrate concentrations until the cells became fully non-responsive. Such cells lost the response to butyrate even as far as the ability to hyperacetylate core histones during butyrate treatment. In contrast, JCA-1 is nearly indistinguishable from DU145 in this respect. Clearly, JCA-1 is a unique resource for investigation of HDAI action. We are working toward publication of this interesting phenomenon, which includes a variety of molecular expression changes surprisingly similar to that seen in cells which manifest an acute butyrate-induced growth inhibition. The work complements that of a number of other investigators who have reported growth control aberrations in PrCa lines and tumors *in situ*.

B. The TSU-Pr1 cell line becomes very dependent upon high cell density when challenged to recover replication ability after butyrate-induced growth arrest. This phenomenon is extraordinary because of the nearly full recovery of replication abilities when treated as a dense culture, yet only approx. 1% survive when treated at low clonal densities. The TSU-Pr1 line has a 20-30% colony forming efficiency (CFE) on plastic regardless of the input cell number, indicating a likely absence of such paracrine signaling requirements under logarithmic growth conditions. Such contact-dependence of cytotoxicity has been demonstrated previously for response to genotoxic agents, survival being improved by culture in a bone stromal cell environment or in the presence of defined growth factors such as IGF1. Our analysis is proceeding initially along similar lines.

Bone stroma-supported culture of PrCa cells from bone biopsies

When resection of a primary cancer fails to prevent the recurrence of metastatic disease, it is our belief that cells at distant sites and in the primary tumor are populated by cells with increased autonomy, no longer requiring interaction with local stroma. Such cells may have greater survival and growth ability in laboratory culture settings. At the start of this project we thought that applying several modifications to the growth of prostate cancer cells from primary tumors would yield at least 2-4 cell lines from 40 trials. The modifications that we had applied successfully to colorectal and breast cancers (delay in passage until paracrine crossfeeding and the retention of cells with decreased adherence to culture dishes) were thought to be sufficient. Needless to say, despite our attention to detail, we obtained no long term cultures and no cell lines, seeing all epithelial cells disappear shortly after the fibroblastoid cells died.

We now believe that cancers with a preponderance of metastatic bone recurrence may continue to require stromal factors of some type, possibly in a presentation typical of bone. Knowledge of the bone microenvironment remains rudimentary or fragmentary even for hematopoietic cells, and the mechanism of prostate and breast cancer metastasis is thought to be largely that of 'homing' or specific adherence. However, empirical use of osteogenic stromal cultures (in which osteoblast differentiation persists for months) may be beneficial not only for improving the proportion of laboratory PrCa cell lines that express PSA, but may also allow definition of the microenvironmental factors essential for support of semiautonomous cancers.

To this end we have:

1. Organized a system for procurement of bone biopsies from patients with advanced prostate cancer. We have 3 enthusiastic clinical coinvestigators who believe that such an aim is consistent with clinical care, and may enhance individualized care in the future. We have IRB approval and seed grant funds.
2. Developed criteria for comparing individual lots of bone stroma cultures (derived from discarded human femur heads resected during hip replacement surgery) using vonKossa and alkaline phosphatase cytochemical stains, and ECM isolation and characterization.
3. Optimized methods for quantifying growth and survival PrCa cell lines in coculture, distinguishing carcinoma from stroma using antibodies to cytokeratin, which are universally expressed by our 6 cell lines. We are also developing methods for short-term tracking (4-6 cell divisions) of seeded PrCa cells using lipid-soluble fluors developed for immunobiological applications.
4. Determined the relative sensitivity PrCa cells to HDAI and protein kinase C activators (PKCA) such as TPA and mezerein, when the cells are cultured in bone stroma, on collagen, in conditioned medium and under standard conditions (plastic substrate and growth medium, with 10-90% of saturation cell densities (see below).

SOW (Statement of work)-related issues

Butyrate-responsiveness comparisons – growth rate and colony formation – We have repeated each of these determinations more than twice for each line. This is being written up for publication, presented in the context of paradigms A and B of the 'Determinants of recovery.....' section above.

Interaction of HDAI and PKCA toward growth arrest and death of prostate cancer cell lines – Although JCA-1 can largely resist initial growth inhibition by butyrate, up to 50% of the cells fail to form colonies after a 2 day exposure. While we have yet to quantitate the loss of survival over time, it appears that dead cells accumulate in the medium at later times of treatment. This was overlooked at first, a result of the impressive disregard of early growth inhibition. Thus, it may be that butyrate-induced cell death is independent of the magnitude of the initial growth inhibition, a prediction which can be tested only in a cell line such as JCA-1. As with the VACO 5 cell line, which is reversibly growth inhibited but also suffers approximately 50% loss of colony formation after 2 days of butyrate treatment, the PKC activators mezerein and TPA synergize to reduce colony forming efficiency nearly 100% despite very little effect of the PKC activators by themselves.

We have returned to the issue of PKCA/HDAI interaction only recently, after the realization that the JCA-1 and TSU-Pr1 cell lines were indeed susceptible to butyrate-induced irreversible growth arrest, and that PKC activators markedly decreased survival in each cell line. Because of differences in timing and lower proportions of cell death attributable to primary apoptosis, it is less likely that these phenomena are synonymous with acute (12-24 hr) apoptosis of VACO 5 and COLO 201 cells effected by butyrate and TPA (12). Rather little

apoptosis is seen during the first 48 hr in butyrate-treated JCA-1, and butyrate depresses replication of TSU-Pr1 cells only in sparse cultures, irregardless of the use of a PKC activator. As discussed above, we are attacking the problem of JCA-1 sensitivity to butyrate-induced death from the point of view that the cells seem to ignore CDKI actions, and will reopen our investigations of PKC interactions once we have better identified likely determinants of this unresponsiveness. Candidate functions for PKC action include heparan sulfate proteoglycan loss (13) leading to extracellular matrix destabilization and/or growth factor receptor desensitization, perturbation of MAP kinase signaling elements and bcl-2 family protein phosphorylation. However, the experiments that we are capable of at this stage, correlative measurements, would likely not yield information as to whether the change was causative. We have data from colorectal cancer cell lines that suggest that proteoglycan losses are important for butyrate-induced commitment to both apoptosis and terminal differentiation, but have no evidence for a role of heparan proteoglycan loss in senescence phenomena, and in fact . We may pursue this in the future in connection with bone matrix effects on survival, as it is our belief that fibrillar collagen complex of bone stabilizes cell surface proteoglycans as one of the trophic actions of bone matrix.

Expression of developmental proteins – Among existing PrCa cell lines, PSA is expressed only by LNCaP. In line with many other investigators, we have found this marker to be expressed at higher intensity and by a larger proportion of the population at 24 hr after treatment with butyrate or TPA. We have a similar study underway with EpCAM and cytokeratin 8, markers expressed by a greater range of PrCa cell lines. EpCAM is expressed by nearly 100% of LNCaP and ND-1, about 50% of DU145 and <1% of JCA-1, TSU-Pr1 and PC-3. The later result, which used conventional fluorescein-conjugated secondary antibodies contrasts with a published report (14). The fact that this protein and cytokeratin 8, which is expressed by all 6 lines, are not limited to LNCaP, suggests that their expression is less likely to be androgen driven, in contrast to PSA, even when butyrate-induced increases are considered (10).

Because of the profound morphological changes after mezerein or TPA treatment of several of these lines, effects that might interfere with interpretation of immunofluorescence results, we feel it necessary to repeat this work to quantitate expression using our digital microscope while in parallel examining a solubilized preparation for immunoblot-reactive protein (i.e., relative activity per mg of whole cell lysate). Each of the preceding markers are important to our project for differentiating PrCa cells from bone stroma, and will thus be presented as preliminary data for an upcoming resubmission of a grant proposal. We plan to use examine the heterogeneity of DU145 expression of EpCAM to examine the possibility of heterogeneous survival or gene expression by this cell population when the cells are either treated with butyrate (or SBHA) or grown on bone stromal culture or 'conditioned' culture components thereof.

We have yet to examine cytokeratins 5/22 and bcl-2 family members, but this goal was proposed mainly in connection with our prediction of success in long term culture of PrCa cells.

P53 functional competence - We have determined the response of each of the 6 PrCa lines to ionizing radiation as a challenge in regard to p21 expression, a widely accepted indicator of p53-dependent transcriptional activity. Only JCA-1 and LNCaP (ATCC) express this competence. (Recent reports suggest that some LNCaP variants are mutant in p53.)

Bcl-2 family expression and phosphorylation - We have done relatively little thus far. We have had some difficulty with bcl-2 immunoblotting, and need to determine the source of the problem (primary antibody reagent?). Analysis of the members mcl-1 and bcl-X (immunoblot semiquantitation) is important both with respect to developmental issues and survival control, and is a priority for completing this study. We plan to quantitate relative expression of each of these proteins by the panel of lines, but may not get beyond that. These proteins may determine the relatively long survival of the cells after commitment to death, but we have considered cell death *per se* to be less important compared to commitment to non-

replication; some of the goals of the original S.O.W. may be misplaced effort given the greater value of mechanistic analysis of themes A and B above.

P53 functional ablation and effect on butyrate-responsiveness - Our original hypothesis was that elements responsible for p53-mediated growth arrest may protect cells from imbalances accruing from HDAI-induced growth inhibition. For example, the HCT116 colorectal cancer cell line is highly tolerant of butyrate treatment, and apparently competent in both p53-mediated hyperexpression of p21 and cell cycle arrest, while VACO 5 and COLO 201 lack this function. The JCA-1 cell line responded to ionizing radiation in that p21 protein accumulated in these cells after induction by 2-10 cGy X-rays. However this cell line continues to replicate after either serum-withdrawal or butyrate treatment despite a rise in p21, an inhibitor of several cyclin-dependent kinases. We suspect, and intend to determine whether these cells slow their growth after irradiation (yet to be tested). Because the ultimate fate of butyrate-treated JCA-1 can be growth arrest and death if treatment is prolonged, this cell line may interest radiation oncologists. The findings that high grade PrCa tumors often exhibit high expression of p21 despite evidence of continuing growth suggests that this cell line may be more representative of aggressive variants of PrCa than we had originally thought.

The LNCaP cell line is also responsive to X-irradiation with p21 induction, and therefore provisionally functional in p53. In contrast to JCA-1 (and many normal cells in culture), this cell line exhibits a profound acute reduction in survival when treated with butyrate. Together with the evidence that the p53 axis of growth/survival control is dysfunctional in JCA-1 (i.e., despite the p21 induction ability), these findings have suggested to us that it may be less useful to embark on an E6 transfectional study than originally projected. In fact, we will likely not make any claims as to the importance of p53 status per se in these responses.

Prostate cancer tissue culture and the efforts to establish more representative cell lines - We consider this a critical effort for the entire field of prostate cancer research. The models available to us for laboratory study seem poorly representative of the disease as a whole. We spent much of our time and resources in the first year trying to nurture cancer cells from resected prostate cancer tissue, and normal prostatic epithelia from contralateral regions of the resected prostate. We used several modifications that we had earlier found useful in culture of colorectal and breast cancer. In not one case out of more than 45 tumors did we obtain cells (epithelia or stroma) that could be passaged beyond 1-3 months. For this reason we have devoted relatively little time over the past year to primary prostate tumors, and much time to developing a normal bone stroma / patient bone biopsy coculture, initially for isolation of metastatic hormone-refractory prostate cancer (see discussion).

Studies not addressed in SOW

Aberrant response of JCA-1 to butyrate-treatment, and its implications for p21 action - While most cell lines respond to butyrate treatment by arrest in G0/G1 and G2 (normal fibroblasts arrest almost exclusively in G0/G1), the JCA-1 cell line shows almost no cycle arrest for about 2 days, and then exhibits a composite of subG1 (apoptotic) G1/G0, and S/apoptotic tetraploid DNA contents. We have thus chosen to focus on the initial responses of these cells to butyrate, i.e.., at a time when the more responsive lines are exhibiting G0/1 and G2 arrest. Whole population cell solubilysates have been examined for gross changes in protein abundance for several cell cycle regulatory molecules. Following on the extensive literature of p21 hyperexpression in butyrate-treated cells (15-18), we find each of the cell lines to exhibit essentially the same pattern of response by overexpressing p21, beginning within hours and persisting for the duration of butyrate exposure (at least as far as 72 hr). Thus JCA-1 resembles the growth-arresting cell lines in that p21 rises progressively between 2 and 24 hr, and falls slightly thereafter. An examination of

individual cells for relative p21 abundance has indicated a high degree of intercellular variability in expression levels in JCA-1, but also in the other lines. We have not yet quantitated these results digitally, but this heterogeneity appears to be a characteristic which can offer insight into butyrate responsiveness. A series of dual-labeling studies, looking for covariability of DNA content (cell cycle-phase) and, especially, pulse-BrdU-incorporation should resolve many of these issues.

While inappropriate for the last few months of the project, it would be interesting to determine whether the expected PCNA/p21 binding interactions were discernible in JCA-1 and the other lines. The results of our single cell p21 profiling /dual labeling will do much to allow us to formulate hypotheses for subsequent testing.

Comparison of response to SBHA and butyrate – Compared to butyrate, the aliphatic bishydroxamate SBHA is a more potent, structurally-distinct, less metabolically labile HDAI. This agent provides results in core histone acetylation pattern and growth responses similar to those of butyrate, suggesting that the unique response of JCA-1 to butyrate is typical of its responses to HDAI in general. The fact that it is not a lipid precursor is a valuable asset for our studies of PrCa cell/bone stroma coculture response to HDAI, in that early work indicated that 3T3 mesenchymal cells respond to butyrate plus glucocorticoid with differentiation toward adipocytes (19). Our preliminary experience suggests that the adipocyte/osteocyte ratio is inversely correlated with PrCa cell growth. Coincidentally, the stroma in cocultures with several of the PrCa lines tend to become markedly less adipocytic.

Identification of factors responsible for butyrate-tolerance of dense PrCa cultures and cocultures of PrCa with bone stroma – The Chung lab has demonstrated that growth factors produced by stromal cells of prostate and bone can enhance the growth of PrCa cells, even those from lines that have been established and passaged extensively in autonomous culture. While these phenomena have been examined in relation to basal rates of growth or hormone action, our interests lie in the effects such factors have on recovery from butyrate-induced growth inhibition, or the initial insult of inhibiting ongoing replication. We are currently trying to optimize the harvest of 'conditioned' matrix, or dish-associated materials. Thus far, we have been unable to effectively substitute these fractions, or bone stromal cell cultures, for moderately dense cultures of TSU-Pr1. We can't yet rule out certain trivial explanations such as altered cell cycle activity and bulk nutrient/oxygen radical concentrations. It remains possible that control of factor lability, post-secretion processing and other complexities like ongoing proteolysis will necessitate use of permeable membranes (e.g.., Transwell™ dishes) or ablational methods for identification.

The prototype of a cancer-related growth factor action, insulin-like growth factor 1 is likely one of the key regulators of autocrine growth support for many cancer cell lines and remains relevant in our studies despite the lack of activity of high dose insulin. The low yield of activity in 'conditioned medium' is not necessarily preclusive of the involvement of either IGF1 or the heparin-binding FGF family members, as both can exist largely as matrix-bound forms with significant turnover.

Establishment of cell lines from metastatic prostate cancers using precultured bone stroma - This is an issue that will appear in the reportable outcomes section. We have initiated an effective program for isolation and analysis of small numbers of prostate cancer cells using bone stroma. Using PC-3 as prototypes for fastidious PrCa cells, and each of the other PrCa lines as relatively autonomous PrCa cells, we have been able to evaluate the butyrate and mezerein responsiveness of small numbers of cells that would otherwise have less than 1% CFE. We are still faced with issues of heterogeneity of colony size, and complexities of drug metabolism mediated by the stromal cells, but our culture system may be an adequate culture

environment for cells adapted to survival in an osteogenic stromal environment. We have IRB approval for patient biopsies at both the Dartmouth-Hitchcock Medical Center and at our VA Medical Center, and have a seed grant from the Beveridge Foundation for materials, salary and a stipend for patients who consent to bone marrow biopsy/aspiration.

Research accomplishments

1. We have assessed 6 of the 12 (?) existing prostate cancer cell lines for response to and recovery from butyrate treatment. These results provide a considerable advance in detail over existing publications, which tend to report the effects on ongoing replication. While the JCA-1 cell line is unique in this regard, the greatest contribution will be in the relative survival of mass cultures of the cell lines TSU-Pr1 and DU-145 or ND-1; one would not predict such a difference in survival given the similarity of initial response.

2. We have identified JCA-1 as having a unique (at least in magnitude) refractoriness to of p21 growth arrest signals and butyrate's growth inhibitory actions. While we need to examine the response by single cells before publishing this phenomenon, it promises to provide a good model for what is now an increasingly well appreciated clinical finding regarding disparity between p21 levels and proliferative activity (20); anaplastic prostate cancers appear to have evaded growth regulatory signals via central inactivation of the downstream components of the p21 signaling network.

3. We have begun to fractionate or otherwise identify the factors associated with moderately-dense TSU-Pr1 cultures that protect the cells, allowing resumption of growth upon removal of butyrate. This cell line has a sufficiently high magnitude of protection that we can anticipate developing a sensitive assay system.

4. As a result of our plunge into stromal cell culture we can now distinguish PrCa colonies from bone-derived cells, and can enumerate both bone nodules and adipocytes, allowing assessment of bidirectional intercellular communications. We have partially completed quantitating HDAI sensitivity of the PrCa lines in such settings, aiming to relate the results to the differential response of the lines to sparse and dense cultures.

Reportable outcomes -

i. Receipt of seed grants to establish bone stroma/prostate cancer cocultures aimed at high frequency isolation of clinical disease for laboratory evaluation and research study.

ii. Two manuscripts in preparation.

a. Publication detailing the unique responses to HDAI of JCA-1 in comparison to the other androgen-independent prostate cancer cell lines.

b. Manuscript addressing the response of prostate cancer cell lines in cultures of varying cell densities, and possible determinants of such recovery.

iii. A functional laboratory with a clinical translational mission.

Anticipated outcomes to be included in final report

Application for grant support to continue work to define intrinsic and extrinsic determinants of recovery from HDAI treatment.

Publication detailing the unique responses of JCA-1 in comparison to the other androgen-independent prostate cancer cell lines.

Manuscript addressing the response of prostate cancer cell lines in cultures of varying cell densities, and possible determinants of such recovery.

Conclusions

Histone deacetylase inhibitors (HDAIs) are a promising new class of experimental cancer therapeutics. Human prostate cancer cell lines (PrCa) are less variable in their responses to HDAIs than are cell lines of human colorectal, lung or breast cancer. While rapid, butyrate-induced apoptosis is less prominent than among cell lines from certain other cancers, cells from 4 of the 6 PrCa lines were nearly fully and irreversibly inhibited in colony forming efficiency, and the other two appeared to be synergistically inhibited if a protein kinase C activator was included during treatment. Responses of the PrCa lines in dense culture were more strikingly different, in that the JCA-1 cell line continued to replicate in the presence of butyrate or SBHA (another HDAI). The other HDAI-tolerant line, TSU-Pr1 could almost fully recover replication abilities upon removal of butyrate when treated at cell population densities near saturation, but was severely inhibited when treated at colony forming assay densities. The mechanisms responsible for these distinct forms of HDAI tolerance are the subject of continuing investigation.

One of the goals of our original proposal was to attempt to establish new cell lines from prostate cancers to achieve a better representation of moderately-well differentiated cancers among available laboratory models. We have been fully unsuccessful at establishing such lines from prostatectomy-derived tumor tissue despite our previous success at establishing cell lines from primary cancers of colorectum and breast. We have begun a program by which metastatic prostate cancer cells, obtained by bone marrow biopsy of persons with advanced disease, can be cultured in close apposition to pre-cultured normal bone stromal cells. Initial results with fastidious human adenocarcinoma cell lines has been highly promising and has been used to quantitate colony forming efficiency of several of the PrCa lines as influenced by HDAI treatment.

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