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In vitro antitumor effect of hydroxyurea on hormone-refractory prostate cancer cells and its potentiation by phenylbutyrate

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Previous clinical trials have suggested that hydroxyurea may possess some activity against prostate cancer. The *in vitro* antiproliferative activity of hydroxyurea was evaluated in three hormone-refractory prostate cancer cell lines, PC-3, DU-145 and PC-3M. Fifty-percent inhibition of growth in all three cell lines required prolonged (120 h) exposure to hydroxyurea at a concentration of approximately 100 μM. Using pharmacokinetic data obtained during the course of a clinical trial of hydroxyurea, we simulated a dosing regimen that would sustain plasma drug concentrations above 100 μM for 120 h (1 g loading dose, followed by 500 mg every 6 h for 5 days in a 70 kg man). Since this dosing regimen is likely to generate an unacceptable degree of myelosuppression, *in vitro* combination studies were conducted with hydroxyurea and phenylbutyrate, a new differentiating agent with no myelosuppressive effects. These studies resulted in a reduction of the hydroxyurea concentration necessary for 50% growth inhibition (50 μM of hydroxyurea plus 0.5 mM of phenylbutyrate). A regimen designed to achieve that hydroxyurea concentration (400 mg loading dose, followed by 200 mg every 6 h for 5 days) should be clinically achievable. Based on these results, this combination deserves further evaluation in patients with stage D prostate cancer.

Key words: Hydroxyurea, phenylbutyrate, prostate cancer.

Introduction

Hydroxyurea, a ribonucleotide reductase inhibitor, is a simple chemical compound ($CH_4N_2O_2$, molecular weight 76.05) that was initially synthesized in the late 1800s.[1] It was later found to produce leukopenia in laboratory animals and subsequently was tested as an antineoplastic agent.[2] At present the primary clinical role of hydroxyurea is in the treatment of myeloproliferative disorders. It is now considered one of the preferred initial therapies for chronic myelogeneous leukemia.[3]

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Hydroxyurea has been evaluated in a number of solid tumors, including malignant melanoma, squamous cell carcinoma of the head and neck, renal cell carcinoma, and transitional cell carcinoma of the urothelium.[4–7] Initial studies appeared promising in several of these diseases, but further investigation has not defined a role for hydroxyurea in any of the standard therapy regimens for solid tumors.

Inasmuch as hydroxyurea is an S-phase cell cycle specific agent, we were surprised that several clinical trials of this drug in hormone-refractory metastatic prostate cancer suggested that it possessed some activity,[8–12] particularly given (i) the slowly progressive nature of the disease and (ii) the schedules of drug administration used in these trials, e.g. once daily to once every 3 days. It seemed unlikely that either the doses or schedules of hydroxyurea administration used in these trials would capture a significant proportion of tumor cells in a susceptible phase of the cell cycle. Table 1 summarizes the reported clinical data regarding hydroxyurea's activity in hormone-refractory prostate cancer. The overall objective response rate is 23% and the frequency of subjective improvement is 36%.[8–12] In order to determine whether there is a pharmacologic rational for further studies of hydroxyurea in hormone-refractory prostate cancer, we evaluated the *in vitro* antiproliferative activiy of this compound against three hormone-refractory prostate cancer cell lines (PC-3, PC-3M and DU-145) and correlated these *in vitro* results with the human pharmacokinetics of hydroxyurea. Because the concentrations of hydroxyurea required for inhibition of cell growth in tissue culture could be achieved in humans only at the expense of severe myelosuppression, we evaluated hydroxyurea's activity in combination with phenylbutyrate, a relatively non-toxic differentiating agent.

Table 1. Clinical trials utilizing hydroxyurea in prostate cancer

Author	Year	*N*	OR	SR	R	HU Dose
Lerner *et al.*[8]	1977	30	63% (19/30)	76% (23/30)	N	80 mg/kg q3d × 6 wk
Kvols *et al.*[9]	1977	5	60% (3/5)	NR	N	3 gm/m^2 q3d
Loening *et al.*[10]	1981	28	14% (4/28)	21% (6/28)	Y	3 gm/m^2 q3d
Mundy[11]	1982	22	36% (8/22)	68% (15/22)	N	80 mg/kg q3d
Stephens *et al.*[12]	1984	69	4% (1/24)[a]	13% (9/69)	Y	3.6 gm/m^2 2d/wk

OR, objective response (stable, partial, complete response); SR, subjective response; R, randomized (N = no, Y = yes); HU Dose, dose of hydroxyurea.
[a]Stable response not reported and only evaluated patients with soft tissue disease.
Response criteria varied between the trials.

Materials and methods

Cell cultures and reagents

Two of the human prostate cancer cell lines (PC-3 and DU-145) were obtained from American Type Culture Collection (Rockville, MD). PC-3M was obtained from James Kozlowski (University of Wisconsin via the NCI Cancer Treatment Screening Program at Frederick, MD). Hydroxyurea was purchased from Sigma (St Louis, MO). Sodium phenylbutyrate was obtained from élan Pharmaceutical Research Corp. (Gainesville, GA). The cell culture medium used was RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin 5000 units/ml, streptomycin 5000 μg and 2 mM L-glutamine (Gibco, Grand Island, NY).

Experimental

The prostatic carcinoma cell lines were propagated in RPMI 1640, which was supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). The cells were grown to 80% confluent monolayers (6×10^5 cells/cm^2) and cultivated in 75 cm^2 flasks (Nunc, Roskilde, Denmark). Cells were harvested with trypsin (0.05%):EDTA (0.02%) (Gibco) solution and counted in a hemocytometer. The cells were then seeded into 96-well microtiter plates (CoStar, Cambridge, MA) at a density of 3000 cells per well in RPMI 1640 medium with 10% FBS and 1% penicillin–streptomycin solution, and reincubated for 24 h to allow for cell reattachment (37°C, 5% CO_2 atmosphere). Hydroxyurea, diluted in tissue culture medium to yield a final well volume of 200 μl, was then added at specified concentrations (0.01, 0.1, 1, 10, 100, 1000, 10 000 and 100 000 μM) and left undisturbed for 120 h in the tissue culture incubator. Control cells were grown in an equal volume of medium. The 3-(4,4-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide (MTT; Sigma) assay was used to estimate cell number.[13] After exposure to hydroxyurea, MTT (20 μl, 5 mg/ml in PBS) was added to the wells, incubated for 4 h and centrifuged at 2000 *g*, and the medium was decanted. Dimethyl sulfoxide (150 μl) was added to each well, the plates were shaken for 30 s and the optical density at 540 nm was determined on a kinetic microplate reader (Bio-Tek EC340 Immunoplate-reader).

The synergistic studies were conducted with PC-3 cells by adding 0.5 and 1 mM of phenylbutyrate to 50 and 100 μM of hydroxyurea. Similar *in vitro* conditions and durations of drug exposure were utilized as described above. Cell numbers were determined by hemocytometer.

Statistical

Statistical analysis was performed using the SAS computer program (version 6.02; SAS Institute, Cary, NC). When comparing the significance of differences in the mean optical density produced at various concentrations of hydroxyurea, the adjusted two-sided Wilcoxon rank-sum test was used. An $\alpha \leq 0.05$ was considered statistically significant. To determine the relationship between hydroxyurea dose and cell survival, the experiments were conducted five times for the PC-3 cell line, and three times for both DU-145 and PC-3M. In each experiment there were six wells per concentration. The experiments to determine the optimal duration of exposure were performed once with 12 wells per fixed concentrations (five plates for both methods).

Results

For all three cell lines there was a dose-dependent decrease in the percentage of cells surviving with hydroxyurea concentrations between 10 and 1000 μM (see Figure 1). At a concentration of 100 μM there was an approximate 50% reduction

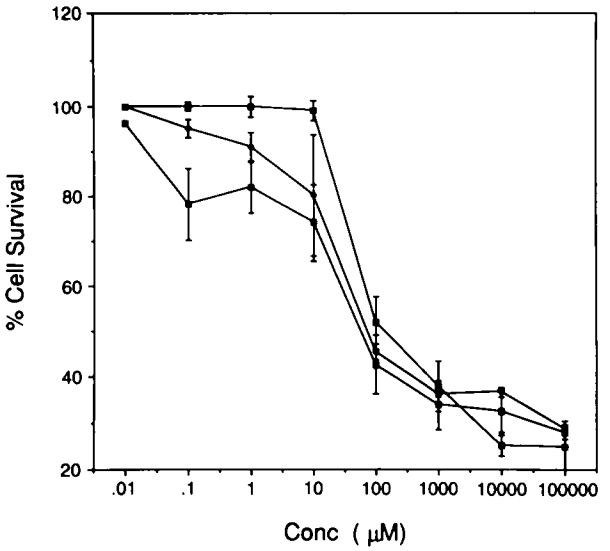

Figure 1. Dose–response curves of hydroxyurea in three prostate cancer cell lines (PC-3, PC-3M and DU-145) compared with the control wells. The IC_{50} for all three cells is approximately 100 μM. ⊡, PC-3; ●, DU-145; ■, PC-3M.

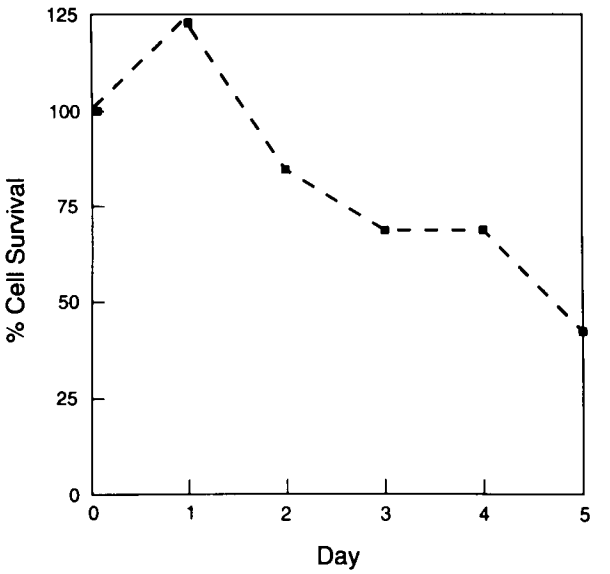

Figure 2. Duration of drug exposure versus survival curve of hydroxyurea (100 μM) in PC-3 cells. The cells were exposed to drug for varying periods of time, but all were grown for a total of 5 days (120 h). Drug exposure was terminated at the various intervals by replacing drug-containing medium with drug-free medium. This experiment did not detect any recovery in cell viability following brief periods of drug exposure. Again, the IC_{50} was only achieved after 120 h of exposure. □, 100 μM.

in cell survival (42.7, 45.5 and 52% for PC-3, DU-145 and PC-3M, respectively), for all three cell lines, compared to untreated cells ($p = 0.016$). Increases in the hydroxyurea concentration above 1000 μM exerted little further effect on the inhibition of cell growth (see Figure 1). At concentrations less than 100 μM hydroxyurea was only cytostatic, but cytotoxicity increased with increasing concentrations above 100 μM, 86% of the total cells were viable at 100 μM and 41.9% at 1000 μM, determined by Trypan blue staining (Gibco) after 5 days of exposure.

Two additional experiments were conducted with PC-3 to investigate the relationship between the duration of hydroxyurea exposure and inhibition of cell growth. In the first experiment cell survival was assessed daily, from 24 to 120 h of exposure, with four different concentrations of hydroxyurea (0.1, 1.0, 10 and 100 μM); the MTT assay was performed immediately upon completion of the specified duration of hydroxyurea exposure. In the second experiment cells were exposed to the same concentrations of hydroxyurea for varying periods of time (24, 48, 72, 96 and 120 h), but grown for a total of 120 h before performing the MTT assay. Drug exposure was terminated at the various intervals by replacing drug-containing medium with drug-free medium. This later experiment was performed to detect the possibility of a recovery in cell growth following brief periods of drug exposure (see Figure 2). In both experiments there was a decrease in the percentage of surviving cells with increasing duration of exposure. There was no evidence of a plateau in the effect by 120 h nor of resurgence of cell growth following drug washout.

With increasing concentrations of hydroxyurea and phenylbutyrate there was a marked alteration in PC-3 cell morphology and growth characteristics. Specifically, cells exposed to hydroxyurea had reduced cytoplasma to nucleus ratio, as well as reduced proliferation. Cells exposed to phenylbutyrate, alone or in combination with hydroxyurea, had an increased cytoplasm to nucleus ratio. Those cells also regained contact inhibition of growth, which suggest a reversion to a more benign phenotype (see Figure 5).

The pharmacokinetic model and model parameters used to describe the disposition of hydroxyurea in humans were derived from concentration versus time data previously reported from clinical trials of this drug.[13–20] Curve-stripping techniques (Abbottbase™ Pharmacokinetic System, version 1.0) applied to the concentration versus time data following a single oral dose of hydroxyurea (80 mg/kg) indicated that these data were best described by a single exponential decay, allowing a single compartment open linear model to be used in pre-

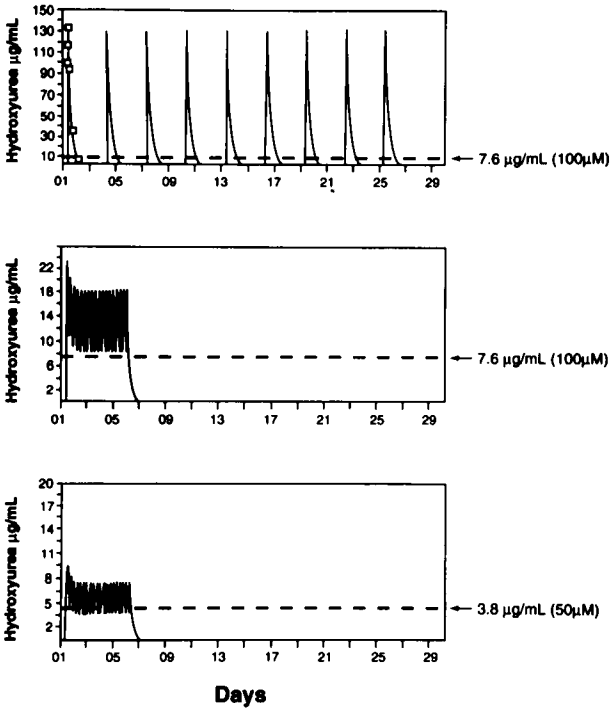

Figure 3. (Top) A plasma concentration versus time simulation of the hydroxyurea dosing regimen employed by Lener *et al.* (80 mg/kg every 3 days) depicted over 30 days. (Middle) A plasma concentration versus time simulation of the hydroxyurea dosing regimen required to produce plasma concentrations above 100 µM for 5 days in an average 70 kg man (1.0 g loading dose followed by 500 mg every 6 h for 5 days) depicted over 30 days. (Bottom) A plasma concentration versus time simulation of the hydroxyurea dosing regimen required to produce plasma concentrations above 50 µM for 5 days in an average 70 kg man (400 mg loading dose followed by 200 mg every 6 h for 5 days) depicted over 30 days.

dicting hydroxyurea's pharmacokinetics. The bioavailability of hydroxyurea has never been precisely determined but is reported to be 'complete',[3] hence we assumed a bioavailability of 100%. Estimates of hydroxyurea's total body clearance were obtained using the trapezoidal rule. Using the average param-eter values derived from this model (volume of distribution, half-life and clearance) we simulated the hydroxyurea dosing regimen used by Lerner *et al.* (80 mg/kg every third day; see Figure 3A). This regimen provides only very brief exposure to hydroxyurea concentrations with *in vitro* antiproliferative activity. A theoretical regimen of a 1.0 g loading dose followed by 500 mg every 6 h for 5 days in a 70 kg man is shown in Figure 3(B); this regimen approximates the conditions required *in vitro* for 50% inhibition of prostate cancer cell growth.

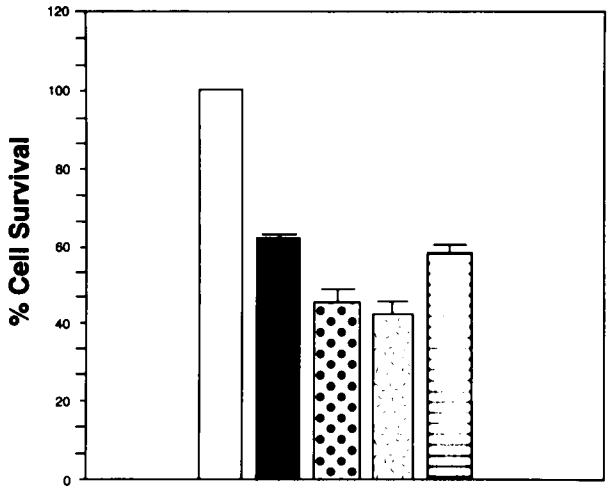

Figure 4. Antitumor activity of the combination of hydroxyurea with phenylbutyrate in PC-3 cells. □, control; ■, PB 0.5 mM; ▨, PB mM; ▫, HU 50 µM/PB 0.5 mM; ▣, HU 50 µM.

Because hydroxyurea plasma concentrations of 100 µM or greater may cause an unacceptable degree of myelosuppression in some patients, we examined the ability of phenylbutyrate to alter the efficacy of hydroxyurea. Hydroxyurea at the suboptimal concentration of 50 µM was combined with phenylbutyrate at concentrations of 0.5 and 1 mM. A 58% reduction in PC-3 cell growth was achieved when 0.5 mM of phenylbutyrate was added to 50 µM of hydroxyurea. As monotherapy, hydroxyurea (50 µM) reduced cell proliferation by only 38% and phenylbutyrate, 0.5 and 1 mM, by 38 and 55%, respectively. Approximately 60 and 80% inhibition of PC-3 growth was combined with 0.5 mM and 1 mM of phenylbutyrate, respectively (see Figure 4).

The growth characteristics and morphology of treated PC-3 cells is shown in Figure 5.

Discussion

The results of previous clinical trials indicate that hydroxyurea might possess activity in the treatment of hormone-refractory prostate cancer. Lerner *et al.* initially evaluated hydroxyurea in 1977 for the treatment stage D prostate cancer.[8] The drug was administered as a single oral dose (80 mg/kg) every third day. A partial objective tumor response was reported in 15 of the 30 patients treated. Subsequent to Lerner's initial study there have been four additional clinical reports of hydroxyurea in hormone-refractory prostate cancer.[9–12] Mundy studied the

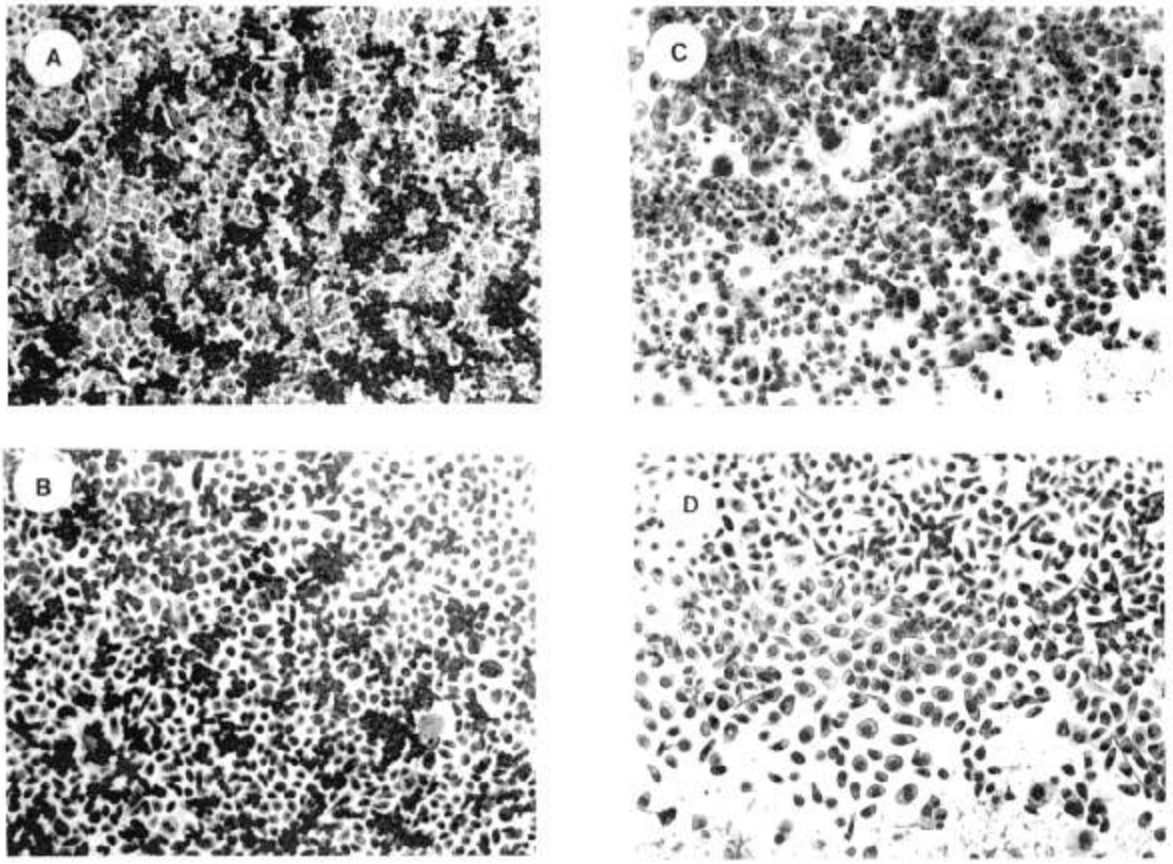

Figure 5. Growth characteristics and morphology of treated PC3 cells. Photographs of PC3 cultured following 5 days of treatment with 50 μM of hydroxyurea (B), 0.5 mM of phenylbutyrate (C) or the combination of 50 μM of hydroxyurea plus 0.5 mM of phenylbutyrate (D). Untreated controls PC3 cells are shown for comparison (A). Note that cells exposed to phenylbutyrate, alone or in combination with hydroxyurea have an increased cytoplasm to nucleus ratio. These cells also regained contact inhibition of growth, indicative of reversion to a benign phenotype (original magnification × 100).

same regimen of hydroxyurea administration (80 mg/kg every third day) in 22 patients failing hormonal therapy and reported improvement in bone pain and performance status in 15 of these patients (68%) within 6 weeks of starting therapy.[11] Kvols *et al.* reported a single partial objective response with hydroxyurea in a group of five patients.[9] The activity of hydroxyurea in hormone-refractory prostate cancer was evaluated in a randomized study carried out by the National Prostatic Cancer Project. In this study 125 patients failing hormonal therapy were randomly assigned to treatment with either methyl-chloroethyl-cyclohexy-nitrosurea (semustine), cyclophosphamide or hydroxyurea.[10] Objective response was observed in 30% of the patients receiving semustine, 35% of the patients receiving cyclophosphamide and 15% of the patients receiving hydroxyurea. Nonetheless, improvement in performance status was greater in the group of patients receiving hydroxyurea (18%, five of 28 patients) than in the other two groups (one of 27 patients for the sermustine group and five of 43 patients for the cyclophosphamide group), and it was equivalent in relieving bone pain. Another randomized, multicenter trial compared the regimen of doxorubicin plus cyclophosphamide to hydroxyurea in 158 patients with stage D prostate cancer.[12] No statistically significant difference in antitumor activity could be demonstrated between the two regimens (objective response rate six of 19 patients versus one of 24 patients, $p = 0.06$). However, response was evaluated only in patients with measurable soft tissue disease, who represent a minority (15%) of the patients with prostate cancer and whose biology may differ from that of patients having disease limited to bone.[21] Unfortunately, comparing the response rates for these trials is im-

peded by the use of heterogeneous, imprecise and vague response criteria at the time the trials were conducted.

If the modest antitumor activity observed in previous clinical trials of hydroxyurea in hormone-refractory prostate cancer was the result of the drug's ability to inhibit DNA synthesis during S-phase, then it is clear that the dosing regimens used in those trials were suboptimal. It is reasonable to assume that the theoretical regimen simulated in Figure 3(B) would undoubtedly result in neutropenia. Because it was unlikely that such a hydroxyurea dosing regimen would be well tolerated *in vivo*, we explored the effect of combining lower concentrations of hydroxyurea with phenylbutyrate *in vitro*. Phenylbutyrate undergoes rapid conversion to phenylacetate *in vivo* by B-oxidation.[22] Phenylbutyrate and phenylacetate have been used in children with urea cycle abnormalities and appears to be well tolerated in high doses.[23] Both agents have been recently identified as antineoplastic agents effecting tumor growth and maturation.[24,25] In addition to causing selective cytostasis, both induce malignant cells to undergo reversions to a more benign phenotype. Our data indicate that a combination of hydroxyurea and phenylbutyrate, each used at cytostatic concentrations, results in significant inhibition of cell proliferation of the PC-3 cell cultures.

In conclusion, our results indicate that a much greater hydroxyurea exposure (i.e. more than 100 μM for at least 120 h), as a single agent, is required for *in vitro* cytotoxic cell death than has been achieved in previous clinical trials. A regimen designed to achieve that concentration would most likely result in unacceptable side effects. However, hydroxyurea in combination with phenylbutyrate may have a clinical role in patients with hormone refractory metastatic prostate cancer. This combination regimen would require a relatively low dose of hydroxyurea (400 mg loading dose followed by 200 mg every 6 h for 5 days, see Figure 3C) to produce a concentration of 50 μM, as well as a reduced concentration of phenylbutyrate. The hydroxyurea dose employed may cause a mild reduction in neurophils, but this reduction should be clinically tolerated particularly if used in combination with granulocyte colony stimulating factor. The adverse effects associated with phenylbutyrate should not be additive (i.e. central nervous system depression) to the myelosuppression associated with hydroxyurea. Based on these results, this combination deserves further evaluation in patients with stage D prostate cancer.

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