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H_2O_2-clearance Kinetics of Crude Catalase Preparations from Prostate Cancer Cell Lines Treated with Physiological and Synthetic Small Organic Ligands of PPAR-γ

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Authors' contributions

Author JKS conceived, performed the experiment and completed the data analysis and the interpretations. He wrote the manuscript under the conceptual advice of author TT.

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Original Research Article

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ABSTRACT

Aim: To determine the differences in H_2O_2-clearance of two prostate cancer cell lines PC-3 and LNCaP treated with peroxisome proliferator-activated receptor gamma (PPAR-γ) ligands 15-deoxy-$\Delta^{12,14}$ – prostaglandin J_2 (PGJ_2) and nafenopin (Naf)
Study Design: Catalase activity in cytosolic protein fractions was determined and the kinetic parameters Michaelis constant K_m, maximum velocity V_{max}, and V_{max}/K_m were used to determine H_2O_2-clearance differences and the presence of isozymes. Western blot of microsomal and nuclear protein fractions were used to determine the induction of PPAR-γ by ligand treatment and cytochrome P450 (CYP450) enzymes.
Place of Study: Department of Experimental Pathology, University of Vienna, Medical School Vienna; part of an ongoing study which started in 2000.

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Methodology: One million cells of cultured PC-3 and LNCaP cell lines were treated with PGJ_2 or Naf for 48 hours. Enzyme kinetics and Western blots were used to confirm differences in H_2O_2-clearance.
Results: PGJ_2 and Naf induced PPAR-γ greater in PC-3 than in LNCaP. V_{max} of PC-3 catalase was increased by PGJ_2 (1.37 fold increase) but not by Naf (0.09 fold decrease) treatment while Naf (1.29 fold increase) but not PGJ_2 (0.26 fold decrease) increased the V_{max} of catalase in LNCaP. The changes in K_m by the two chemical substances were statistically not significant ($0.2 < p < 0.6$) for both PC-3 (mean $K_m = 0.59 \pm 0.05$ mM) and LNCaP (mean $K_m = 1.2 \pm 0.036$ mM) cultures. Changes in V_{max} led to equal magnitude of change in V_{max}/K_m but which was again not statistically significant ($p = 0.6$) between PC-3 and LNCaP cultures (control and treatment cultures). Lower K_m always led to a lower V_{max} and the vice versa but V_{max} and V_{max}/K_m values were always higher for LNCaP than their corresponding catalase from PC-3 cultures.
Conclusion: The marked catalase activity of PC-3 and LNCaP, the high expression of drug metabolizing CYP450 enzymes and the different PPAR-γ induction kinetics in prostate cancer cells observed in this study can be explored for the modulation of redox status of prostate cancer cells with PPAR-γ ligands.

Keywords: PPAR-γ; catalase; H_2O_2-clearance; prodrugs; oxidative stress.

1. INTRODUCTION

Intracellular pH, oxidative and reductive stress, are emerging as promising markers of improving drug action in target cells [1,2,3]. Small and big organic molecules in the cell that modulate oxidative and reductive states of the cell can serve as biomarkers upon which redox compounds can be generated as pro-drugs [4]. Pro-drugs are designed on the rationale that a higher concentration of the drug can be concentrated in the target cell compared to the administration of the drug itself [2]. Reactive oxygen species and oxidoreductases that control their levels are important biomarkers and activators, which can be used to predict the accumulation and transformation of pro-drugs into active drugs in targeted cells [4]. By similar strategy, the reducing conditions of tumor cells have led to the design of bio-reductive pro-drugs based on the specific oxidoreductases of tumor cells for conversion into active drugs [3]. PPARs are transcriptional regulators of enzymes involved in fatty acid and glucose catabolism, which can be considered as biomarkers of increase oxygen tension and oxidative stress in view of the increased generation of reactive oxygen species and molecular oxygen utilization involved in catabolism of fatty acids and glucose in the cell [5]. In addition, scientific reports have indicated the transcriptional regulation by PPARs of antioxidant enzymes catalase, superoxide dismutase and glutathione reductase; members of a group of oxidoreductases that control the clearance of reactive oxygen species of the cell [5]. Among these antioxidant enzymes catalase is the most dominant, since it is expressed by all cell types [6]. Increased malignancy and inflammatory conditions have been shown to be associated with increased expression of peroxisome proliferating activated receptors (PPARs) [7,8]. The marked difference in physiology between the most studied prostate cancer model cell lines, the androgen-independent and the most metastatic PC-3 compared to the androgen-dependent and least metastatic LNCaP and DU-145 cell lines, present an opportunity to develop experimental protocols to study their responsiveness to drugs in *in vitro* settings. In our previous study [9] we demonstrated greater induction of PPAR-γ expression in PC-3 cell line than in LNCaP cell line by peroxisome proliferator nafenopin with a concomitant increases in percent catalase activity. This investigation was therefore aimed at comparing the kinetics of catalase-based H_2O_2 clearance of PC-3 and LNCaP cell lines based on Michaelis-Menten steady state model as shown in equation 1 below:

$$V = V_{max}[S]/\{K_m + [S]\} \quad (1)$$

V=velocity of catalysis, V_{max} = maximum velocity, [S] = substrate concentration and K_m is the Michaelis-Menten constant.

H_2O_2 clearance can serve as a determinant of the redox status of prostate cancer cells, in the context of PPAR-γ induction by ligands comprising peroxisome proliferators [5] and physiological agents [10].

2. MATERIALS AND METHODS

2.1 Chemicals

Specific antibodies (primary and secondary) for PPAR-γ, CYP450, NADPH-reductase and Lowry reagent for protein determination were purchased from Amersham, UK. Protein inhibitors phenylmethylsulfonyl fluoride, leupeptin, aprotinin and calpain were obtained from Sigma-Aldrich, UK. Hydrogen peroxide, clofibrate, nafenopin (Naf), 15-deoxy-$\Delta^{12,14}$ – prostaglandin J_2 (PGJ_2), organic solvents and buffer components were purchased from Merck, Germany. All buffers were prepared with doubled distilled water and adjusted to 0.04 molality and pH 7.4 for preparation of cell culture media.

2.2 Cell Culture

Prostate cancer cell lines PC-3 (ATCC CRL 1435), LNCaP (ATCC CRL 1740) and DU-145 (ATCC HTB 81) were seeded at one million cells per 10 mL of culture medium (RPMI 1640; Gibco, BRL) supplemented with 5% fetal calf serum (Gibco, BRL) and 100 µL/mL of penicillin-streptomycin (Gibco, BRL). Cultures were performed in 75-mL culture bottles in incubator (Cytoperm, Heraeus) at 37℃ in air containing 5% carbon dioxide at 95% humidity.

2.3 Treatment of Cell Lines with PPAR Ligands

Inocula of one million cells from each of PC-3 and LNCaP cultures were transferred into separate 10 mL of fresh culture medium and subcultured every other day for one week. After one week the cultures were treated with 10 µM of the ligands nafenopin, and the 15-deoxy-$\Delta^{12, 14}$ – prostaglandin J_2 (PGJ_2). Duplicate cultures were set for each treatment for each cell type. Cultures were harvested after 48 hours of incubation. Ligands were dissolved in DMSO giving DMSO concentration of 0.1% in each culture. Control cultures were treated with only DMSO. Cultures were also set for DU-145 cell line and for comparing the induction kinetics of PPAR-γ by nafenopin and clofibrate in PC-3 cultures.

2.4 Preparation of Cytosolic, Microsomal and Nuclear Protein Fractions

Spent media were aspirated and cells were washed briefly with Ca^{2+}, Mg^{2+} - free phosphate-buffered saline (Gibco, BRL). Cell monolayers were removed with trypsin-EDTA solution and separated from monolayer by centrifugation at 2000 rpm. 10 million cells from each treatment culture were suspended in 1 mL ice-cooled Hepes buffer, pH 7.9 containing 10 mM Hepes, 10 mM KCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.2 mM Na_3VO_4 and regime of protein inhibitors; 1 mM phenylmethylsulfonyl fluoride, 100 mM leupeptin, 10 mg/mL aprotinin and 10 mg/mL calpain. Cells were lysed by freeze and thaw in liquid nitrogen with intermittent vortexing. Lysed cells were centrifuged at 1500 rpm for 5 minutes at 4℃ to remove debris. The supernatant were subjected to centrifugation at 40,000 rpm for 5 minutes at 4℃. The resulting supernatants were stored as the cytosolic protein fraction. The residuals were stored in 50 µL aliquots in 10 mM $KHPO_4$ pH 7.4 containing 20% glycerol at -80℃ as the microsomal protein fraction. For preparation of nuclear protein fraction lysed cells were centrifuged at 12,000 rpm for 10 minutes and the residues were subjected to further homogenization by freeze and thaw in 20 mM Hepes pH 7.9, containing 1.5 mM $MgCl_2$, 420 mM NaCl and 0.2 mM EDTA. The resulting suspension was clarified at 15,000 rpm at 4℃ for 15 minutes. The supernatant obtained was diluted in equal volume of 20 mM Hepes buffer pH 7.9 containing 100 mM KCl, 0.2 mM EDTA and 20% glycerol and stored in 50 µL portions at -80℃ prior to their use. Protein was determined using Lowry method [11].

2.5 Western Blot Protein Analysis

80 µg of nuclear (PPAR) or microsomal (CYP450) proteins were electrophoresed by SDS-PAGE in 12% polyacrylamide gel [12]. The electrophoregram was visualized with 1% Coomassie blue stain in methanol. The protein bands were electroblotted onto nitrocellulose membrane (0.2 µm pore size, Bio-rad). The membranes were blocked in 5% non-fat dry milk in phosphate buffered saline containing 0.05% Tween at 4℃ for 2 hours and washed extensively in the same buffer. The membranes were incubated with the primary antibody 1:1000 (rabbit polyclonal anti-human PPAR-γ antibody) in PBS-Tween buffer at 4℃ for 18 hours. The membranes were subsequently washed with PBS-Tween buffer. The membranes were further submerged in PBS-Tween buffer containing secondary antibody 1: 5000 (horseradish peroxidase conjugated anti-rabbit IgG) for 2 hours at room temperature. Membranes were washed in PBS-Tween for several minutes and

the bands of interest were detected on X-ray films after treating membranes with enhanced chemiluminiscence solution (ECL kit Amersham, UK)

2.6 Catalase Activity

10 μL of cytosolic protein fraction was added to 1.8 mL of 50 mM phosphate buffer pH 7.0. 1.2 mL of 30 mM H_2O_2 in 50 mM phosphate buffer was added to initiate the reaction at 30℃. Absorbance change (ΔA_{240}) was recorded at 240 nm for every 10 seconds against blank solution. ΔA_{240} was calculated for each sample as the mean of three absorbance changes. ΔA_{240} min^{-1} was converted into μmole of H_2O_2 decomposed per minute using the molar extinction coefficient of 40 $mM^{-1}cm^{-1}$ of H_2O_2 at 240 nm. Activity was recorded as percentage of catalase activity in the control cell culture (100%). 10 μL of each of the cytosolic fractions was used to assess the kinetic parameters of catalase clearance of H_2O_2. Activity was determined at different H_2O_2 concentration ranging from 0.05 mM to 1.0 mM. Kinetic constants K_m, V_{max} and V_{max}/K_m were computed for catalase activity for each culture experiment.

2.7 Statistics

Microsoft Excel for Mac 2011 was used for statistical and kinetic analyses. Data were expressed as the mean ± SEM and Student's *t*-test were used in the comparison of different experiments. *p*-value was also performed on Excel worksheets to determine significance. The parameters K_m and V_{max} were extracted from data by mathematical manipulation using excel program.

3. RESULTS

3.1 Time-dependent Induction of PPAR-γ

Two types of synthetic peroxisome proliferators nafenopin and clofibrate gave different patterns of PPAR–γ induction in PC-3 cell cultures as shown in Fig. 1.

After 24 hours of clofibrate treatment the induction of PPAR-γ has reached its peak whilst its level was similar to that of the control culture after the same period of nafenopin treatment. At the 48th hour the level of PPAR-γ band of cells treated with clofibrate has depleted to the level of the control band when in the nafenopin-treated cells the band intensity was more than the constitutive band of the control culture. A lower molecular weight variant PPAR-γ2 was induced after 48 hours of nafenopin treatment as shown in Fig. 1.

3.2 Immunoblot Analysis of PPAR-γ

Western immunoblot analysis depicted the marked differences in responsiveness of PPAR-γ to induction by peroxisome proliferator nafenopin and a physiological ligand PGJ_2 in PC-3 and LNCaP cultures. In Fig. 2 PC-3 appeared to be more responsive to nafenopin and PGJ_2 treatment than LNCaP. The expression of the high and low molecular weight variants of PPAR-γ show equal prominence in LNCaP compared to PC-3 where the level of the low molecular weight variant is visible only under induction.

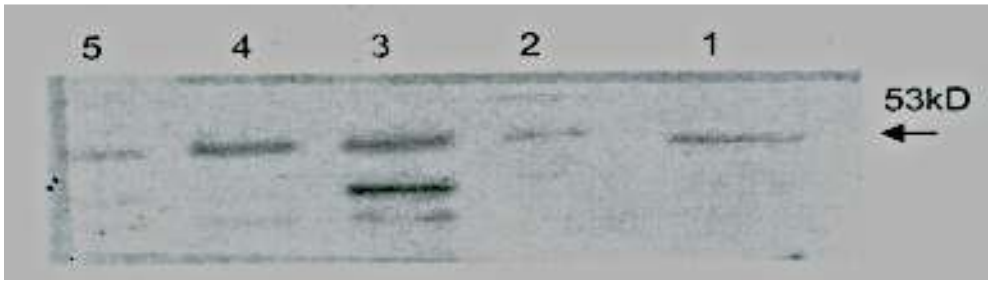

Fig. 1. Induction of PPAR-γ in PC-3 cell line

Time dependent preliminary studies showed that nafenopin can maintain PPAR-γ induction longer (24 hours =2, 48 hours=3) than clofibrate where induction reached its peak earlier (24hours=4, 48 hours=5). Lower molecular weight variant of PPAR-γ was induced by nafenopin and not by clofibrate Inductions were also compared to control PC-3 culture (48 hours=1)

3.3 Changes in Catalase Activity in PC-3 and LNCaP Cell Lines under Ligand Treatment

The clearance of H_2O_2 by catalase from each of the treatment cell line was compared to their corresponding controls. As shown in Table 1, both PC-3 and LNCaP were affected by the different inducers of PPAR-γ on the basis of their ability to deplete H_2O_2.

3.4 Expression of CYP Enzymes in Prostate Cancer Cell Line

High levels of cytochrome P450 enzymes CYP 1A1, 1A2, 2B1, 2E1 and NADPH-reductase enzyme were detected in the prostate cancer cells PC-3, LNCaP, and DU-145. The levels of the oxidoreductases were different for each cell type as shown in Fig. 3.

There were high expressions of CYP 1A1, 1A2, 2E1 and NADPH-reductase compared to CYP 2B1 by the different prostate cancer cell types. Their constitutive difference may affect their levels of activation and contribution to the total oxidative status of the cell through their different drug transformation activities.

4. DISCUSSION

In general cancer cells generate higher amounts of reactive oxygen species than their normal cell counterparts. This has been the basis of the development of pro-drugs, such as the amino-ferrocenes, which remain inactive in non-malignant fibroblasts but become activated in human promyelocytic cancer cells [13]. Metabolic generation of reactive oxygen species as by products of normal cell respiration may be required for control and regulation of the body's physiological systems. However excessive leakages from peroxisomal and mitochondria units; the main sites for energy metabolism may result in univalent reduction of O_2 and consequently the over production of reactive oxygen species as shown by the scheme below:

$$O_2 + e^- \rightarrow O_2^{\cdot-} + e^- \rightarrow H_2O_2 + e^- \rightarrow HO\cdot + e^- \rightarrow H_2O \quad [14]$$

superoxide anion — hydrogen peroxide — hydroxy radical

Activities of enzymes such as xanthine oxidase, uricase, amino oxidases, pyruvate oxidase, glucose oxidase and other aerobic dehydrogenases can also increase the levels of H_2O_2 in the cell [6,15,16]. Major enzymes involved in the clearance of H_2O_2 in the cell are catalase and the peroxidases such as glutathione peroxidase. However, catalase was chosen for this study since, it is expressed by all type of cells and have both oxidative as well as peroxidative activity which is comparable to the peroxidases [17]. In our previous studies, we showed the high responsiveness of PC-3 cells to Naf treatment which led to higher induction of PPAR-γ1 and -γ2 compared to LNCaP [9]. This study has shown a similar pattern of induction when the two cell lines were treated with PGJ_2 a physiological ligand of PPAR-γ. The intrinsic kinetic characteristics K_m and V_{max} of crude catalase extracted from treatment and control cell lines show the different modes of H_2O_2 clearance of the two prostate cancer cell lines. K_m was higher (1.2±0.036 mM) for LNCaP than PC-3 (0.59±0.05 mM), and the ligands appeared to have no effect on the K_m as shown in Table 1 ($0.2 < p < 0.6$). However, for the V_{max} the difference was significant; both control and treated PC-3 cultures gave lower values than similar cultures of the LNCaP ($p = 0.0034$). H_2O_2-clearance measured in this study as V_{max}/K_m for the crude catalase preparations was however statistically not different for the control and treated cultures of each of the two cell lines ($p = 0.6$) even though for the two cell lines a higher V_{max} led to increase in V_{max}/K_m as shown in Table 1. Furthermore V_{max} was lower for the Naf-treated PC-3 than the control PC-3 (0.09 fold decrease) and PGJ_2-treated PC-3 gave higher V_{max} than the control (1.37 fold increase). The reverse was observed in LNCaP where Naf-treated cells gave higher V_{max} than the control (1.29 fold increase) and the V_{max} was lower for the PGJ_2-treated LNCaP than the control culture (0.26 fold decrease). The seemingly lower values of V_{max} for the PC-3 culutres (control and treatment) compared to same cultures of LNCaP suggest that at a given saturating levels of H_2O_2 PC-3 will retain higher levels of residual H_2O_2 than LNCaP and become more sensitive to higher levels of H_2O_2 than LNCaP due to its lower K_m. LNCaP cells may be more tolerant to higher H_2O_2 levels than PC-3 cells due to its higher K_m value. Again at any given rate of H_2O_2 degradation PC-3 catalase will required lower concentration of H_2O_2 than LNCaP catalase and at any given H_2O_2 concentration below their respective K_m PC-3 catalase will have higher activity than LNCaP catalase (9). The study has also shown higher V_{max} for the constitutive catalase in LNCaP cells than in PC-3 cells, which could explain the differences in responsiveness

of PC-3 and LNCaP cells to PPAR-γ ligands and to the induction of catalase expression. The above analyses support the findings that PC-3 retains more H_2O_2 than LNCaP, which might explain certain PC-3 aggressive characteristics [10]. The different kinetics of H_2O_2 clearance demonstrated in this study may imply different isozymes of catalase expressed by the two prostate cancer cell lines. The catalase C-262T polymorphism for example, is related to the development of prostate cancer [18]. This study further suggests that different levels of PPAR-γ ligands may define the H_2O_2 levels in the prostate cancer cells, which can be exploited for selective *in vitro* activation experiments of pro-drugs. The differences in PPAR-γ induction kinetics by nafenopin and clofibrate, both alpha-subtituted carboxylic ligands and the high constitutive levels of lever-type P450 enzymes provide a wide variation of activation/deactivation ligands and the plethora of approaches that can be considered in conducting such experiments.

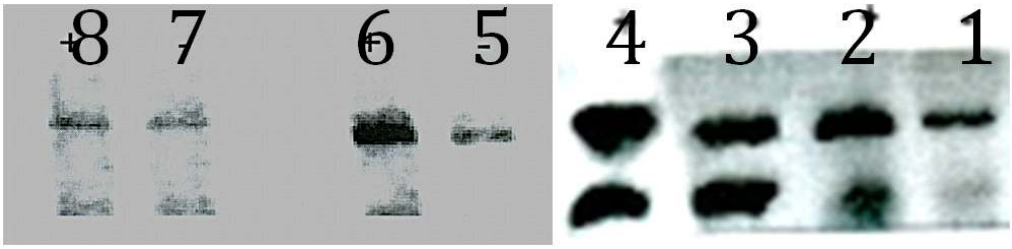

Fig. 2. Immuno-analysis of PPAR-γ expression in prostate cancer cell lines. 10 µM each of the ligands Naf (2, 4) and PGJ_2 (6, 8) were administered to cells. The induction of PPAR-γ compared with the untreated cells (1, 3, 5, 7)

Note: Bands for the Naf cultures were magnified x400, contrast and picture background were changed to make the bands more visible

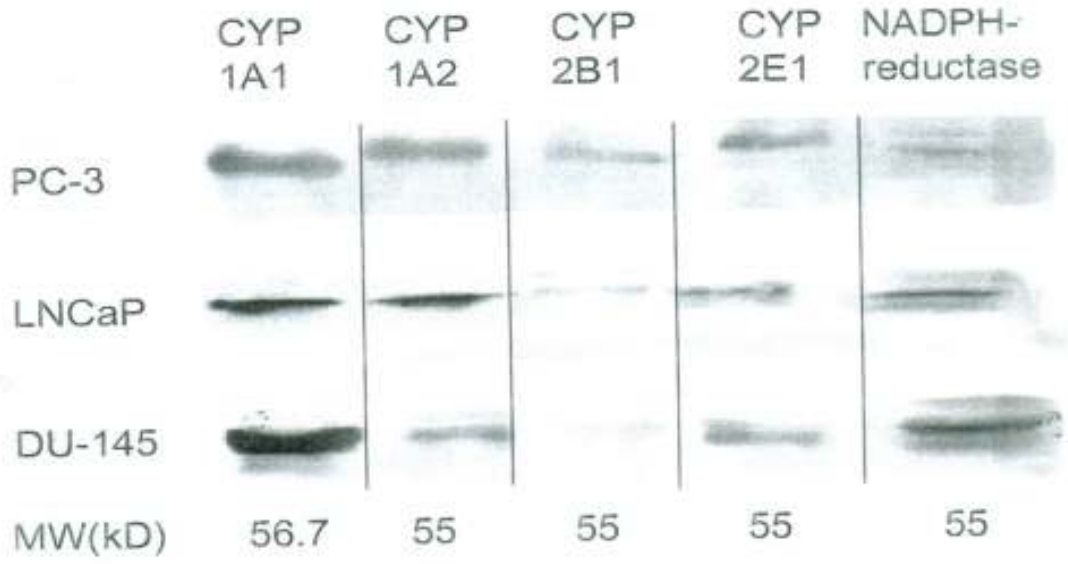

Fig. 3. Electrophoregram of selected CYP P450 in prostate cancer cells

CYP 1A1, 1A2, 2B1, 2E1 and NADPH-reductase were identified by Western-blot assays in cell lines PC-3, LNCaP and DU-145

Table 1. Determination of kinetic parameters K_m and V_{max} of catalase activity in PC-3 and LNCaP cell cultures. The mean K_m was used to calculate for H_2O_2 clearance V_{max}/K_m for treated and untreated cell lines

	K_m (mM)	V_{max} μM/min	V_{max}/K_m min^{-1}
	0.59±0.05∗		
PC-3 (control)	0.61	141	0.24±0.03
PC-3 (Naf)	0.53	131(0.09 FD)	0.22±0.01
PC-3 (PGJ_2)	0.60	192(1.37 FI)	0.33±0.04
	1.2±0.03∗		
LNCaP (control)	1.3	368	0.31±0.009
LNCaP (Naf)	1.4	476(1.29 FI)	0.40±0.014
LNCaP (PGJ_2)	0.96	263(0.26 FD)	0.23±0.014

∗There was no significant change in the kinetic parameter K_m in the drug treated and control cell lines by student's t-test for each cell type (p between 0.2-0.6). However, V_{max} values for PC-3 cultures were highly significantly different from LNCaP (p = 0.0034) and these have been used to calculate the H_2O_2 clearance V_{max}/K_m values as shown in Table 1. K_m and V_{max} values are averages from duplicate determinations. FD = fold decrease, FI = fold increase

5. CONCLUSION

Decades of clinical and basic science trials have attempted to improve outcomes of prostate cancer aggressiveness using treatment strategies based on cell oxidative status. Our current study has equally demonstrated the prospects of experimenting PPAR-γ ligands for the modulation of catalase levels and thereby altering the H_2O_2 clearance status of prostate cancer cells PC-3 and LNCaP. The K_m and V_{max} data in totality suggest the existence of different isozymes of catalase in PC-3 and LNCaP but taking the H_2O_2-clearance (V_{max}/K_m) values of their control and treatment cultures together were found to be statistically not different. Thus, different K_m and V_{max} values but similar of V_{max}/K_m values of catalase-catalyzed reaction could be used to confirm catalases from different sources as isozymes. The modulation of H_2O_2 clearance can serve as an attractive strategy of a selective concentration of cancer therapeutics in prostate cancer cells. The study also confirms earlier finding that prostate cancer progression from androgen-dependent and least metastatic to androgen-independent and most metastatic is accompanied by altered antioxidant status.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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