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# TACKLING RADIORESISTANCE OF HYPOXIC CELLS BY METABOLIC MODULATION OF BIOENERGETICS – A $^{31}$P MRS STUDY ON PERFUSED EHRLICH ASCITES TUMOR CELLS

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**Abstract :** In an attempt to overcome resistance of hypoxic cells to radiotherapy, the combination of a hematoporphyrin derivative (Hpd) and 2-deoxy-D-glucose (2-DG), a promising radiomodifier, was evaluated by assessing the alterations in phosphorylated metabolites and bioenergetics induced in perfused Ehrlich ascites tumor (EAT) cells, using Phosphorus-31 Magnetic Resonance Spectroscopy ($^{31}$P-MRS). By reducing flow rate of perfusion, a relatively hypoxic condition of tumor was simulated. Changes in bioenergetics levels induced by the combined treatment of Photosan®, a Hpd, and 2-DG, under reduced perfusion conditions were more pronounced. Significantly higher uptake of 2-DG and irreversibility of the reduction in cellular bioenergetics induced by the combined treatment, observed under simulated hypoxic conditions, might have considerable implications in optimizing tumor radiotherapy using 2-DG as an adjuvant. These result also suggest the usefulness of this technique to easily simulate hypoxic conditions of tumors *in vitro* that could be used for rapid *in vitro* pharmacological evaluation of promising therapeutic strategies.

**Key words :** $^{31}$P MRS, perfused cells, agarose gel-cell threads, bioenergetics, hypoxia simulation, pre-pharmacological screening, 2-Deoxy-D-glucose

## INTRODUCTION

The Pharmaceutical scientists are on a continual look out for improved screening methods for prompt evaluation of potential therapeutic strategies aimed at reducing the expenditures involved in drug development. MR Spectroscopy (MRS) offers unmatched possibilities for obtaining multiparametric non-invasive information on the therapy induced effects under normal and perturbed metabolic conditions (1, 2). One of the major limiting factors for standardization of tumor therapy protocols, is the individualistic nature of tumors (3). Generally, the microvasculature in solid tumors is not able

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to keep pace with the unlimited rate of tumor growth resulting in inadequate and heterogeneous blood perfusion (4). Knowledge about oxygenation of tumors and its importance in influencing radiotherapeutic responses is crucial to the efforts to improvise the radiotherapy procedure (5–7). Hypoxic cells *in vitro* and in animal tumors *in vivo* are reported to be around three times more resistant to radiation-induced killing compared to normal cells (8, 9). Current methods for dynamically evaluating the treatment response under simulated hypoxic conditions of tumors are sub-optimal (10).

Research on bioenergetics of cancer cells is of great relevance in oncology since the differences observed between energy metabolism of normal and cancer cells may be exploited for designing therapeutic modalities with better selectivity (2, 11). As the strategy of modulation of energy-linked DNA repair (12) by 2-deoxy-D-glucose (2-DG) has a clear rationale for selective radiosensitization of cancer cells (13), its use as an adjuvant to radiotherapy is currently under clinical evaluation (14). Under euoxic conditions, 2-DG induced inhibition of repair processes has been shown to be partly reversible after removal of treatment (15). In a human cerebral glioma cell line (BMG-1), it has been observed that inhibition of DNA repair could be made irreversible in the absence of respiratory metabolism (16). However, similar effects could also be achieved by modulation of the pharmacokinetics of 2-DG (17). Hematoporphyrin derivatives (Hpd) have been shown to affect the energy yielding pathways by inhibiting cytochrome-c-oxidase, thereby reducing oxidative phosphorylation in tumor cells (18), this in turn increases the glucose utilization and glycolysis (19).

The aim of the present study was to simulate nutritional conditions of tumor using agarose–gel cell thread perfusion technique and to explore the feasibility of evaluating the bioenergetic response of Hpd and 2-DG combination under comparatively hypoxic conditions.

## METHODS

### Chemicals

Low-gelling-temperature agarose (Type VII) for immobilization of cells and 2-DG were obtained from Sigma Chemical Co., USA. A commercial preparation of Hpd, Photosan®-2 (P-2), was obtained from Seehof Laboratarium GmbH, Munich, Germany.

### Cell-culture and sample preparation for MR spectroscopic studies

Murine Ehrlich ascites tumor (EAT) cells, F-5 strain maintained and grown in cell culture in '$A_2$' medium (15, 17) containing 20% v/v horse serum, were used in these experiments. EAT cells (which have a doubling time of ~12 h) were kept in quasi-continuous culture by reducing the cell concentration from ~$8x10^5$ cells/ml to $5x10^4$ cells/ml in fresh nutrient medium, on every alternate day. From a suspension of exponentially growing population, cells were inoculated to the fresh nutrient medium (60 ml) in disposable culture flasks (Nunc, Holland) and incubated in a water

jacketed $CO_2$ incubator under standard conditions (5% $CO_2$, 310 K, saturated water atmosphere) to a cell density of approximately $10^6$ cells/flask (after 48–52 h of growth).

For the MRS studies, the cells were immobilized by embedding in low-gelling-temperatures agarose and a life support system was set up to perfuse these gel-cell threads in a custom designed perfusion assembly (11). Approximately $2.0 \pm 0.3 \times 10^8$ cells in the stationary phase were harvested and washed with Hepes buffer (Serva, Heidelberg, Germany). Cell pellet obtained by centrifugation (1000 rpm, 277 K, 10 min), was resuspended in buffer to give a volume of 2 ml. To this, 1 ml of 2.1% low-gelling-temperature agarose solution at 310 K was added and gently mixed. The cell-gel suspension was extruded under mild pressure from a coil of pre-cooled tygon tubing (i.d. = 0.5 mm) into a screw capped 10 mm precision glass NMR tube (Wilmad Inc., USA), containing 2 ml of perfusate, i.e., cold $A_2$ medium containing 5% v/v of horse serum. The cell-gel threads in the tube were lightly compacted by the hollow teflon insert and continuously perfused with perfusion medium at ambient temperature at a flow rate of $1.7 \pm 0.1$ ml/min, using a peristaltic pump. The perfusion solution flows from the opening of the inlet tygon tube (internal diameter 0.5-mm) near the bottom of the NMR tube, through the gel-cell threads, and the outflow was directed through opening in the insert to the outlet tubing. About 25-30 ml of the perfusate was discarded after switching over to changed medium before resorting to recirculation. The perfusate was replenished every 3 h.

**Simulation of nutritionally deficient condition of tumor**

Aglycemic hypoxia was induced by stopping the supply of perfusate to the cell gel matrix. Relatively hypoxic conditions of tumor were simulated by lowering the rate of flow of perfusate from 1.7 to 0.9 ml/min using peristaltic pump.

**MR spectroscopy of perfused EAT cells**

MR spectroscopic measurements were performed on Bruker's AM 270 or AM 500 NMR spectrometers at the Institute for Biophysical Chemistry, University of Frankfurt, Germany. Sample tube was not spun during measurements. Phosphorus-31 spectra were recorded at spectrometer frequency of 109.355 MHz and 202.46 MHz (for AM 270 and AM 500 spectrometers respectively) at 16 K data points with relaxation delay of 1.5 sec. Deuterium oxide (7%) was added to the perfusate for providing deuterium lock to affirm long-term stability of the magnetic field during perfusion. The studies were conducted at $310 \pm 2$ K without spinning. The free induction decays (FIDs) were subjected to an exponential line broadening of 20 Hz before Fourier transformation. Two superimposable control spectra were acquired to ensure the metabolic stability of the cells before administering drug(s) treatment. Drugs were added aseptically to the sterile perfusate. During time course studied, full-width-at-half-maximum (FWHM) did not change significantly and spectra were acquired under identical conditions. Relative concentrations of the metabolites of interest were compared from

the peak height on the premise that equivalent samples are being compared.

## RESULTS

A typical P-31 MR spectrum of perfused EAT cells is shown in Fig. 1. The peak positions are expressed in parts per million with reference to the 85% orthophosphoric acid resonance. According to chemical shifts and spin coupling values reported in literature (17, 20), major peaks can be assigned to phosphomonoesters (PME)– predominantly phosphoethanolamine (PE) and phosphocholine (PC), inorganic phosphate (Pi), phosphodiesters (PDE) – viz. glycerophosphoethanolamine (GPE) and glycerophosphocholine (GPC), and the three phosphorus atoms of adenosine triphosphate (γ-, α- and β-ATP).

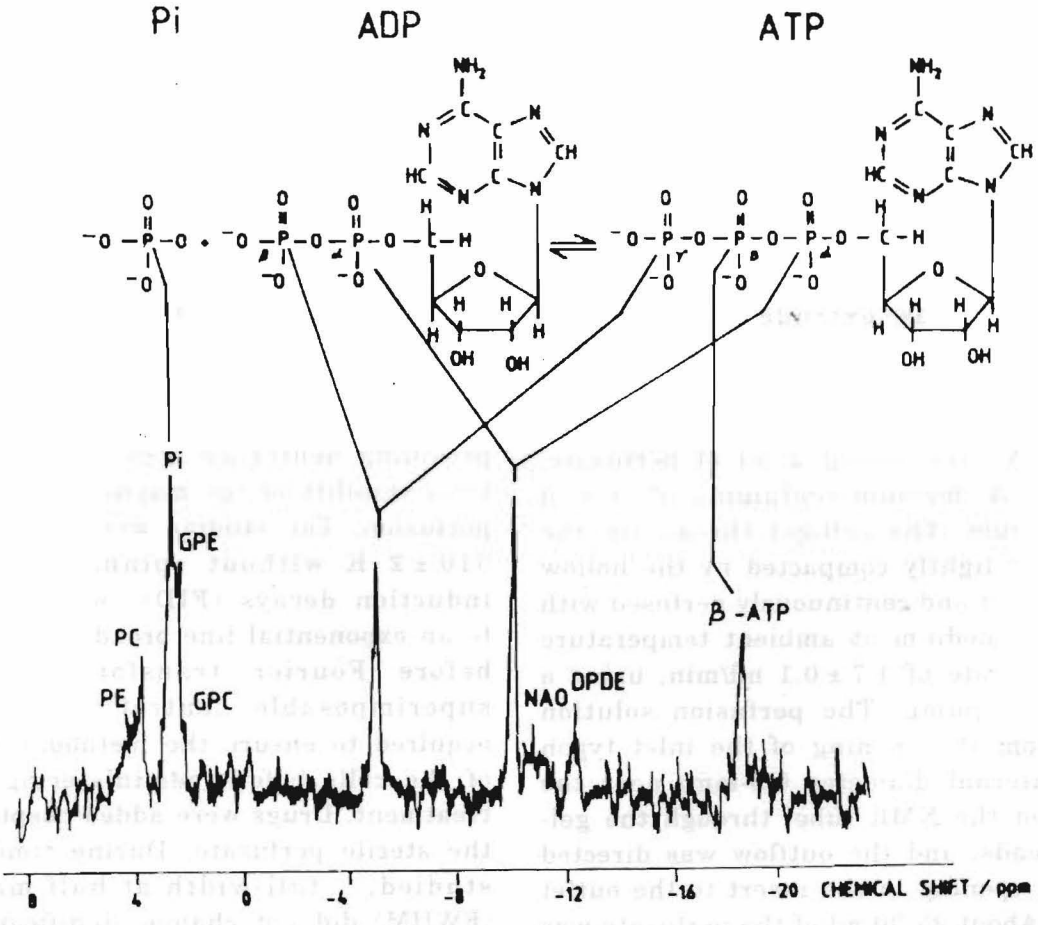


Fig. 1: A representative P-31 NMR spectrum of metabolically active Ehrlich ascites tumour cells. The measurements were recorded on AM 270 spectrometer operating at 109.3 MHz. [NS = 550 scans , RD = 1.5s. Peak assignments: PE, phosphoethanolamine; PC, phosphocholine; Pi, inorganic phosphate; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine and γ-, α- and β-ATP, the three phosphorus atoms of phosphates of adenosine triphosphate.]

The time course of the changes in the P-31 spectra of EAT cells induced by changing the perfusate from a glucose-containing perfusion medium to a medium containing equimolar concentrations (24.9 mM) of 2-DG and glucose (2-DG/G ratio = 1) for a period of 4 h (Fig. 2A) and switching back to perfusate without drug thereafter for another 4 h, are shown in Fig. 2(B). The pH dependent signal at 4.64 ppm, corresponding to 2-deoxy-D-glucose-6-phosphate (2-DG-6-P), began to rise in ca. 20 mins. Highest levels of 2-DG-6-P are achieved at about 130 ± 10 min (flow rate of perfusion = 1.7 ml/min). Detectable amount of 2-DG-6-P was clearly visible even after 4 h of washing off the treatment. The β-ATP reduced to about 50% within 2 h of 2-DG perfusion and remained around this level till 2-DG perfusion continued. The level remained low or reduced further even after 1 h of removing 2-DG treatment.

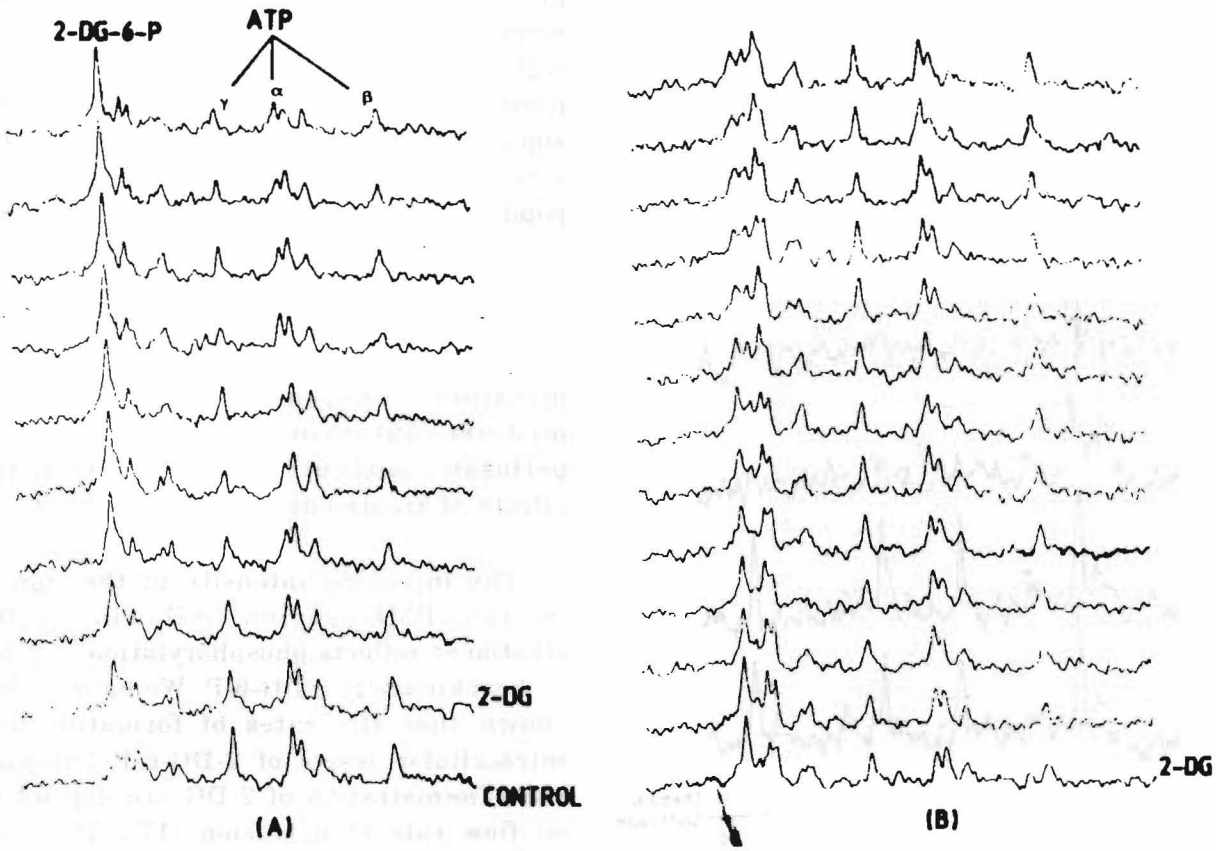


Fig. 2: Time course in P-31 MR spectra of EAT cells showing the changes on phosphorylated metabolites: (A) induced by changing the perfusate from glucose containing medium to a medium containing equimolar concentration of 2-DG perfused for 3 h, (B) 4h-washout kinetics after perfusion with 2-DG for 4 h.

Cellular bioenergetics status (β-ATP/Pi) was monitored before, during and after inducing tumor anoxia/hypoxia by stopping/ reducing the supply of the perfusate. During anoxia, a progressive decrease in the β-ATP resonance was observed alongwith continuous increase in inorganic phosphate signal with upfield shift in its position (data not shown). After about 45 minutes of stopping the perfusate flow, the ATP was no longer visible. Representative spectra showing the effects of P-2 (30 µg/ml) and 2-DG (24.9 mM) administration after pretreatment with P-2 (30 µg/ml) for 1 h, under simulated hypoxic conditions (flow rate of perfusion = 0.9 ml/min) are shown in Fig. 3. The effect of combination of energy profile was very drastic (β-ATP peak merged with the 'noise' and was no longer visible) and irreversible as compared to studies carried out under normal perfusion conditions.

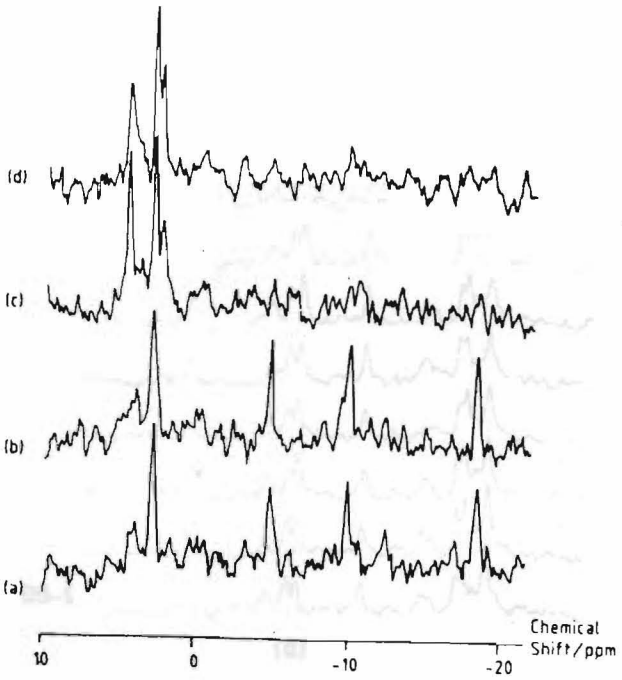


Fig. 3 : Representative P-31 spectra showing the effects of P-2 and 2-DG combination at slower rate of perfusion (Flow rate = 0.9 ml/min) [(a) control; (b), P-2 (30 µg/ml) perfusion for 1 h; (c) after 4 h treatment with P-2 (30 µg/ml) and 2-DG (2-DG/G = 1); (d), reperfusion with perfusion medium without any drugs for 4 h].

## DISCUSSION

The prerequisites of cell perfusion study are to ensure that normal metabolic events remain unhampered. Therefore, substrates and nutrients need be continuously provided and waste products removed simultaneously. Using the agarose gel-cell thread perfusion technique, it is possible to apply a variety of therapeutic simulations, and dynamically measure in situ biochemical changes in the cell population. The drugs could be perfused along with the perfusate through the gel-cell threads while inside the spectrometer and their fate and the effects induced could be dynamically monitored. Replacing the perfusion medium-containing drug with normal perfusate (without drug) tapered off the effects of treatment.

The increased intensity in the signals in the PME region following 2-DG treatment reflects phosphorylation of 2-DG by hexokinase to 2-DG-6-P. We have earlier shown that the rates of formation and intracellular levels of 2-DG-6-P following the administration of 2-DG are dependent on flow rate of perfusion (17). The time course of changes in β-ATP to inorganic phosphate (β-ATP/Pi) ratio has been taken as a measure of cellular energy status. Under hypoxic conditions (Flow rate of perfusion = 0.9 ml/min), the $t_{max}$ of 2-DG-6-

P was reported to be achieved much earlier (80 ± 10 min.) even though the extent of accumulation was lower, and the decrease in energy profile was more acute and sustained (17). At reduced flow rate many resonance also shifted to lower frequencies.

From amongst different metabolic modulators screened for sequential/ concomitant use with 2-DG, the synergistic combination of 2-DG and P-2 that are currently being evaluated individually in various clinical trials (14, 22), was advocated as a safe and effective modality worth further evaluation (17, 21). Hpd treatment has been reported to cause induction of metabolic activation of tumors (23, 24). Upon concurrent administration of P-2 and 2-DG after 2 h pretreatment with P-2, the higher rate of accumulation of 2-DG-6-P observed, confirms increased glycolytic flux in presence of Hpd, in agreement with the intracellular 2-DG-6-P accumulation pattern observed in BMG-1 cells in the presence of 2 mM of KCN (16). Avid uptake and phosphorylation of 2-DG after P-2 pretreatment could be explained on the basis of increased glucose uptake and glycolysis (17, 23, 25, 26) as a result of reduced in vivo respiratory capacity. Furthermore, almost negligible recovery of the energy profile was observed, even 4 hr after removing the treatment. On the basis of this information, it is hypothesized that the combination will preferentially render the tumor more susceptible to radiotherapy by improving delivery of 2-DG to target tissues resulting in drastic effects on bioenergetics. This hypothesis of the potentiality of P-2 and 2-DG combination as adjuvant to radiotherapy, has been duly substantiated in EAT and BMG-I cells *in vitro* using conventional radiobiological techniques (23). Recently, corroboration of above findings is also validated in a murine tumor model (27), which confirm that the drugs exert biochemical changes in animal and human tumor models in the same way as anticipated keeping the expected variables in mind.

In mild hypoxic conditions simulated in perfused cell model, the lower pH milieu is contemplated to further potentiate the effects of 2-DG. However, the specific contribution of lowering of flow rate and 2-DG induced effects on decrease in pH was not investigated as it involved a number of complicating factors of fluid dynamics also. Increased bioavailability of 2-DG resulted in substantial fall in the levels of ATP as the utilization of ATP exceeds the rate of synthesis with almost no recovery after drug treatment was stopped. ATP levels, cell survival, and DNA breaks repairs have been reported to be closely related in EAT cells (15). The diminished ATP synthesis appears to be due to inhibition of oxidative phosphorylation under nutritionally deficient conditions. Thus, the use of P-2 and 2-DG combination under reduced flow rate of perfusion appears very promising for targeting the hypoxic radioresistant fraction of tumors. The generally inhibited energy flow following combined treatment with P-2 and 2-DG under simulated hypoxic conditions is expected to result in the further increased manifestation of the radiation induced damage in the event of

use of these drugs as adjuvant to radiotherapy.

In conclusion, the present study demonstrates that the bioenergetics studies in the simple tumor model using in vivo MRS technique of perfused immobilised cancer cells can substantially improve the rapidity of drug screening by drastically shortening the time lag in carrying out evaluation of appropriate energy-linked metabolic modulators to be used as adjuvant for optimizing tumor therapy. There are major advantages of carrying out pre-pharmacological studies by MRS of intact cells since samples act as their own controls thus minimizing the biovariations, and that cells do not have to be discarded following an experiment and may be used repeatedly to study the time course (11). Furthermore, the studies could be conducted under well-defined and well-controlled conditions that generally cannot be achieved and experimentally maneuvered in the intact organism. Cell-gel threads with appropriate manipulations of the composition and the rate of flow of perfusate could be used as convenient models for simulating some of the nutritional and metabolic conditions in tumors. The cell-gel threads can, therefore, be visualized as the artificial tumor tissue, the inlet tygon tubing of the perfusion assembly could be correlated to the blood vasculature of tumor, and the drug treatment by this technique could be practiced as for intra-tumoral injection. Thus, MRS of perfused cells portrays in improvement in expenditures of time, animal resources, and statistical power over conventional radiobiological and pharmacological methods.

Simulation of the nutritional and oxygenation condition of tumor with easy manipulations could thus be aptly utilised in development and evaluation of effective sensitizers for radio/chemoresistant hypoxic cells. MRS studies of metabolic modulator induced alterations in pharmacokinetics of promising drugs is hence expected to open a new chapter in metabolic engineering with lot of interesting clinical implications. Nonetheless, the results should be carefully confirmed in higher animal models using either MRS or conventional assay techniques before exploiting to human situations.

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