Effects of 6-aminonicotinamide and 2-deoxy-D-glucose combination on the bioenergetics of perfused Ehrlich ascites tumour cells as monitored by $^{31}$P-MR spectroscopy

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Received 14 February 2000; accepted 14 June 2000

The niacin antagonist, 6-aminonicotinamide (6-AN) is currently being evaluated in preclinical trials as a potential radiosensitizer. 2-Deoxy-D-glucose, a glucose antimetabolite is another promising differential radiomodifier. In this paper we report the effects of these antimetabolites on changes in bioenergetics of perfused immobilised Ehrlich ascites tumour (EAT) cells to predict their usefulness in energy-linked modification of radiation response. $^{31}$P-MRS has been used to monitor cellular energetics as reflected in the ratio of P-phosphate of ATP to inorganic phosphate ($\beta$-ATP/Pi) and accumulation of 6-phosphogluconate/2-deoxy-D-glucose-6-phosphate in these cells. We have earlier shown that treatment with 2-DG results in a decrease in the energy profile of perfused EAT cells. Presence of 6-AN alone induces a progressive decrease in the $\beta$-ATPPi ratios. The impaired energy status observed after 6-AN treatment prompted us to probe its utility in potentiating the effects of 2-DG. The combination of 6-AN and 2-DG sharply reduces the signal attributed to $\beta$-ATP to the noise levels. There was no recovery in energy profile even upto 12 hr after perfusion with drug-free medium. Since metabolic depletion of ATP is known to inhibit the post-irradiation DNA repair processes and induce apoptotic cascade, the combination may be of considerable clinical importance for optimizing the efficacy and selectivity of tumour radiotherapy.

Understanding the regulation and control of cellular responses to radiation is essential for exploring potential avenues for radiomodification in radiotherapy practice. ATP is a major cellular energy source in the repair of radiation induced DNA and non-DNA damage. Any interference with the production of ATP thereby, affects the ultimate radiation response. ATP supply in tumour cells can be selectively decreased by modulating the glucose metabolism then the differential inhibition of repair of radiation induced damage in normal and cancer cells is conceivable. As the strategy of perturbation of energy metabolism by 2-deoxy-D-glucose (2-DG) has a clear rationale for selective radiosensitisation of cancer cells, its use as an adjuvant to radiotherapy is currently under clinical evaluation. Combination of ionizing radiation with 2-DG provides a unique opportunity to differentially enhance the radiation damage in cancer cells and at the same time preventing radiation injuries to normal tissues. The inhibition of DNA repair induced by 2-DG under euoxic conditions has been observed to be reversible. Since 2-DG is removed from the blood circulation rather rapidly, combining it with appropriate metabolic modulators of the energy yielding pathways is a promising approach for diminishing reversibility of the effects of 2-DG on bioenergetics.

Radiation response has also been suggested to be dependent on pentose phosphate pathway (PPP) activity. Postirradiation increase of PPP activity in human lymphocytes has been demonstrated. Red blood cells, which are deficient in glucose-6-phosphate dehydrogenase, have been shown to be more sensitive to radiation. Inhibition of pentose pathway could alter the ultimate radiation response by inhibiting repair processes as ribose-5-phosphate and NADPH production will be affected. The oxidative part of the pentose phosphate shuttle is a critical metabolic pathway in some tumour cells and might prove to be a sensitive target for site-directed drug attack. 6-Aminonicotinamide (6-AN), a niacin antagonist, is currently being evaluated in preclinical trials as a potential radiosensitiser. The impaired energy status after 6-AN treatment is expected to po-
tentiate the alterations in energy profile induced by 2-DG. Thus, it is expected that the combination of 6-AN and 2-DG will accelerate the effectiveness of radiotherapy by inhibition of the energy dependent repair processes. Preliminary results have been presented\(^3\).

Materials and Methods
Cell-culture and sample preparation for NMR studies
Murine Ehrlich ascites tumour (EAT) cells F-5 strain, maintained and grown in cell culture\(^15\) were used in these experiments. The cells were grown in suspension culture in 'A2' medium containing CO\(_2\), 310 K, saturated water atmosphere) to a cell density of approximately \(10^6\) cells/flask (after 48-52 hr of growth). For NMR studies, the cells were immobilised by embedding them in low-gelling-temperature agarose, Type VII (Sigma Chemical Co., USA) and a life support system was set up to perfuse these cell-gel threads\(^26\). Approximately \(2.0 \pm 0.3 \times 10^8\) cells in the stationary phase were collected and washed with Hepes buffer (Serva, Heidelberg, Germany). Cell pellet obtained by centrifugation (1000 rpm, 277 K, 10 min), was resuspended in buffer to make up the volume to 2 ml, to which 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 A2 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 (flow rate = 1.7 ± 0.1 ml/min) with perfusion medium at an ambient temperature.

NMR spectroscopy of perfused EAT cells
NMR spectroscopic measurements were performed on Bruker's AM-270 NMR spectrometer at the Institute for Biophysical Chemistry, University of Frankfurt, Germany. \(^31\)P spectra were recorded at spectrometer frequency of 109.35 MHz 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. Experiments were conducted at 310 ± 2K without spinning. The free induction decays (FIDs) were subjected to an exponential line broadening of 20 Hz before Fourier transformation.

Drug treatment
Drug treatment was carried out only after acquiring two identical control spectra to ensure metabolic stability of the cells. 2-DG and 6-AN were procured from Sigma Chemical Co., USA. Drugs were added aseptically to sterile perfusate. About 50 ml of the perfusate was discarded after switching over to changed medium before resorting to recirculation.

Results
In the \(^31\)P-NMR spectrum of perfused EAT cells 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 reported in literature\(^15\), major peaks can be assigned to phosphomonoesters (PME), inorganic phosphate (Pi), phosphodiesters (PDE), and the three phosphorous atoms of nucleoside triphosphate (\(\gamma\) -, \(\alpha\)- and \(\beta\)-NTP). The \(\gamma\)-NTP peaks overlap with \(\beta\)-phosphate of nucleoside diphosphate (NDP). The \(\alpha\)-NTP peak may contain contributions of \(\alpha\)-phosphate of NDP together with other compounds such as oxi-
dized and reduced NAD/NADP. The β-NTP resonance represents nucleoside triphosphates alone, constituting the most appropriate spectral parameter for ATP, although UTP, GTP, and CTP may also contribute to this resonance. Alterations in the major metabolites of EAT cells were observed to be induced by changing the perfusate from a glucose-containing perfusion medium to a medium containing equimolar concentrations of 2-DG and glucose (2-DG/G ratio = 1). 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. 19 min, and the levels of ATP decreased gradually. 2-DG administration caused a decrease in Pi for about 1 hr, followed by a slow but continuous increase. β-ATP reduced to about 50% within 2 hr of 2-DG perfusion and remained at this level till 2-DG perfusion continued. The levels remained low or even reduced further after about 1.5 hr of removal of 2-DG treatment. The build-up of 2-DG-6-P was monitored as a function of time. Detectable amount of 2-DG-6-P was clearly visible even after 4 hr of washing off the treatment. However, upon removal of 2-DG treatment the energy profile recovered as shown in Fig. 2.

Following treatment with 6-AN (0.02 mM), a new peak was observed at 4.72 ppm after ~ 1 hr which has been attributed to 6-phosphogluconate according to chemical shift value reported in the literature and excluding all other possible phosphorus containing metabolites of the glycolysis and pentose phosphate pathways in that region (Fig. 3). The β-ATP/Pi ratio progressively decreased at a slower rate till about 2 hr. When 2-DG (12.45 mM) was given in combination with 6-AN (0.02 mM) for 4 hr, the β-ATP peak decreased sharply (merged with the 'noise' by ~1.7 hr) as shown in Fig. 4. Moreover, there was practically no recovery in energy profile even after 12 hr perfusion with fresh medium. The signals of 6-PG and 2-DG-6-P could not be clearly distinguished and resolved due to the proximity of their chemical shift position.

**Discussion**

The glucose antimetabolite, 2-deoxy-d-glucose (2-DG)
DG), interferes with the cellular energetics in several ways. It competitively inhibits glucose transport inside the cell and is phosphorylated to 2-deoxy-D-glucose-6-phosphate (2-DG-6-P), which inhibits glycolysis by blocking phosphohexose isomerase, thereby suppressing the conversion of glucose-6-phosphate to fructose-6-phosphate. Upon 2-DG treatment, the rate of energy flow has been observed to be considerably reduced in cancer cells. 2-DG preferentially gets trapped inside many tumour cells, which have higher rates of glucose transport, increased activity of hexokinase and decreased glucose-6-phosphate dephosphorylation rates. Thus, 2-DG may selectively sensitize cancer cells that depend on aerobic glycolysis, while protecting bone marrow stem cells from 2-DG's cytotoxic effects.

The nicotinamide analogue, 6-aminonicotinamide, because of its antimitochondrial properties is considered a potential modulator to the effects of irradiation. It has been shown to accelerate the effects of radiation in vitro. In RIF-1 cells, it was reported to enhance the response of tumour cells to radiation, in vivo and in vitro. Several mechanisms for ATP depletion have been postulated. This nicotinamide analog competes with nicotinamide in pathways utilizing NAD(P) and is incorporated into pyridine nucleotides (NAD and NADP) in place of nicotinamide to give the corresponding NAD(P) analogue, i.e., 6-aminonicotinamide adenine dinucleotide phosphate (6-ANAD(P)). Formation of such pyridine nucleotide analogues depletes the tissues of NAD and NADP, besides inhibiting the enzymatic reactions in which pyridine nucleotide plays a part as coenzymes. 6-ANAD(P) competitively inhibits NAD(P) requiring processes especially the pentose phosphate pathway (PPP) enzyme, 6-phosphogluconate dehydrogenase which is an important step in the synthesis of the coenzyme NAD, NADPH and ribose unit, required for biosynthesis and DNA repair. 6-ANAD(P) cannot be reduced either chemically or enzymatically and consequently it acts as a potent inhibitor of glycolysis, the oxidative portion of the pentose-phosphate shuttle, and mitochondrial oxidative phosphorylation. Accumulation of 6-phosphogluconate (6-PG) in the EAT cells within 1-2 hr indicates inhibition of 6-phosphogluconate dehydrogenase, one of the key enzymes of PPP. New peak observed downfield to PME region at 4.72 ppm following 6-AN treatment has been attributed to 6-PG on the basis of chemical shift value reported in literature. Accumulation of 6-PG clearly suggests that the oxidative part of the pentose phosphate shuttle is a critical metabolic step and is a sensitive target for site-directed drug action. PPP provides ribose-5-phosphate for nucleic acid synthesis and NADPH for reduction reactions involving glutathione reductase and ribonucleotide reductase (which reduces ribonucleotide phosphates to deoxyribonucleotides during DNA synthesis). 6-PG is an intermediate metabolite in the pentose phosphate pathway and it is also a substrate of the second 6-ANAD(P), a sensitive enzyme in the shuttle pathway. Koutcher and coworkers observed that 6-AN singly caused significant inhibition of glycolytic flux but had no detectable effect on PPP. 31P-NMR studies of perfused RIF-1 cells indicated that a 4 hr of exposure to 6-AN was sufficient to cause significant accumulation of 6-PG.

2-DG in combination with 6-AN inhibits the bioenergetics of EAT cells to the extent that the recovery is practically not observed even 12 hr after withdrawal of treatment and perfusion with drug-free medium. The mechanism of action of 6-AN in combination with 2-DG of is not known but modulations of energy metabolism and inhibition of production of ATP have been suggested as a possible mechanism. At cellular level, increase in the rate-limiting enzymes of glycolysis, such as hexokinase, pyruvate dehydrogenase and phosphofructokinase, have been observed in tumour cells with high glycolytic rates. In brain tumours higher activity of pentose monophosphate shunt enzymes has been found in comparison with the normal brain tissue. Thus, as malignant transformation increases, tumour cells possibly upregulate the membrane glucose transporter proteins and increase the enzymes activity associated with the nonoxidative as well as the oxidative metabolic pathways of glucose. These alterations in neoplastic cells may explain the enhanced glucose utilization by tumour cells as compared with normal cells. In view of the increased dependence of the energy supply of tumour cells on aerobic glycolysis, 2-DG blocks glucose utilization and glycolysis in tumour cells whereas 6-
AN as an antimetabolite of NAD synthesis, affects the NAD(P)-dependent oxidoreductase reaction in glycolysis and PPP. NADPH supply was compromised by inhibition of PPP. The secondary inhibition of glycolysis is apparent from the time dependent decrease of ATP and the simultaneous increase in the supply was compromised by inhibition of PPP and glycolytic pathways. Depletion of ATP has been directly implicated as a critical determinant in apoptosis, and is, therefore, an important target for complementary enhancement of radiotherapeutically induced cell death. On the basis of above findings it is predicted that repair of potentially lethal damage induced by gamma irradiation may be inhibited to a greater extent if both 6-AN and 2-DG are used in combination with radiotherapy.

Acknowledgement

The authors express their gratitude to Prof W Pohlit, Prof H Ruterjans and Dr H Hanssum of the University of Frankfurt, Frankfurt/M., Germany, and to Director, INMAS for providing the necessary facilities. The work was carried out as a part of an Indo-German collaborative project.

References

2 Jain V K & Pohlit W (1972) Biochym Biophys A 8, 254-263
6 Brown J (1962) Metabolism 11, 1098-1112
7 van Steveninck J (1968) Biochim Biophys Acta 165, 386-394
18 Roberts W, Kartha M & Sagone A L (1979) Radiat Res 79, 601-610
28 Yushok W D & Gupta R K (1980) Biochem Biophys Res Commun 95, 73-81
33 Woodward G E & Hudson M T (1954) Cancer Res 14, 599-605
38 Jain V (1996) Indian J Nuclear Medicine 11, 8-17
44 Martin D S, Dietrich L S & Shits M E (1957) Cancer Res 17, 600-604
46 Varnes M (1988) Natl Cancer Inst Monogr 6, 199-203