Optimization of tumour radiotherapy: Part V—Radiosensitization by 2-deoxy-D-glucose and DNA ligand Hoechst-33342 in a murine tumour

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Radiosensitizing effects of combination of a minor groove DNA ligand, Hoechst-33342, with the glucose analogue and inhibitor of glycolysis, 2-deoxy-D-glucose (2-DG) have been investigated in Ehrlich ascites tumour (EAT) bearing mice following focal irradiation of the tumour with 60Co gamma-rays. Treatment-induced tumour growth delay and tumour free animal survival were evaluated as parameters of radiation response. Focal irradiation of the tumour with a single fraction of 10 Gy induced a moderate delay in tumour growth but did not lead to complete regression in any of the tumours. Intravenous administration of H-342, 1 hr before irradiation enhanced radiation-induced growth delay in a dose dependent manner. Complete regression of the tumour was observed only at a dose of 10 mg/kg body wt, leading to a cure (tumour free survival for more than 100 days) rate of 55%. Administration of 2-DG (2 g/kg body wt; iv) immediately before irradiation significantly enhanced radiation-induced growth delay and resulted in a cure rate of 45%. In combination with this dose of 2-DG (2 g/kg body wt), H-342 at a lower dose (5 mg/kg body wt) significantly enhanced the cure rate to 66%. H-342 or 2-DG given alone or in combination at the doses investigated here did not show any significant effects on the unirradiated tumour.

The success of conventional procedures presently employed in tumour radiotherapy is limited by (a) the presence of intrinsically radioresistant and repair proficient subpopulations of cancer cells and (b) morbidity due to damage to the normal tissues at higher therapeutic doses. Therefore, approaches that enhance the induction of radiation damage and/or inhibit repair processes differentially in cancer cells should improve the efficacy of tumour radiotherapy. Induction as well as repair of DNA lesions, considered to be most important factors in cellular response to injury caused by ionizing radiation, can be influenced by modulation of several physico-chemical as well as biological parameters such as DNA and chromatin structure, tissue oxygenation, presence of antioxidants as well as optimal flow of metabolic energy.

Our earlier work based on the energy linked modification of radiation damage, has shown that the glucose analogue and glycolytic inhibitor 2-deoxy-D-glucose (2-DG) when co-administered with radiation, selectively inhibits the post-irradiation repair processes in cancer cells, thereby enhancing the radiation-induced cytogenetic damage and cell death. Under similar conditions, 2-DG protects the normal cells, by reducing the fixation processes. 2-DG induced inhibition of strand break joining, unscheduled DNA synthesis (repair synthesis) and the repair of potentially lethal damage (PLDR) have been demonstrated in cancer cells. Pre-clinical studies in mice have also shown that, administration of 2-DG just before irradiation leads to the sensitization of tumours, resulting in growth delay as well as enhancement of animal survival. Phase I clinical trials with 2-DG in human cerebral glioma patients have shown that the combined treatment is well tolerated with minimal late radiation damage to the brain.

The DNA ligands, bisbenzimidazole Hoechst 33258 (H-258) and its analogue Hoechst 33342 (H-342) bind selectively in AT rich regions of the DNA minor groove and have been shown to protect DNA against radiation damage in aqueous solutions and cells. H-342, has higher lipophilicity due to the ethoxy substitution on the 4-phenyl ring and is, therefore, more permeable across cell membrane as compared to H-258. Recently, we observed that, both H-342 and H-258 could provide significant protection against whole body irradiation in mice at non-toxic doses. Considerable reduction in the damage to the haemopoietic system (cytogenetic damage in the bone

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marrow cells) and longer animal survival were observed at sublethal as well as lethal doses of radiation. Furthermore, the combination of H-342 and 2-DG provided a significantly higher degree of protection as compared to the effects of these agents administered alone.

In vivo studies in tumour bearing mice have shown that the intravenously administered H-342 binds largely to the nuclear DNA in euoxic cells and less to the hypoxic tumour cells, probably due to diffusion limited accessibility of H-342 in hypoxic regions. Possibly therefore, the hypoxic tumour cells would not be protected to a significant extent by H-342. Hence, the present studies were undertaken to investigate the radiomodifying effects of the combination of H-342 with 2-DG in tumour bearing mice. Radiation-induced growth delay, tumour regression and animal survival were investigated as end points.

Materials and Methods

Chemicals — Hoechst-33342 (H-342) and 2-deoxy-D-glucose (2-DG) were obtained from Sigma Chemical Company (USA) and used without any further purification. All other chemicals were of analytical grade and obtained from E-Merck, India or Qualigens (Giaxo), India.

Mice and tumour transplants — The inbred Swiss strain 'A' male mice (10-12 weeks) used in these studies were obtained from the Institutes' central animal facility and weighed 20-25 g at the time of tumour implantation. They were provided with water and standard mouse food (Liptin, India) ad libitum. The Ehrlich ascites tumour (EAT) cells (strain F-3) obtained from Institute for Biophysics, University of Frankfurt, Germany were maintained by serial passage of tumour cell suspension in the peritoneal cavity of the mice. All experiments were conducted according to the guidelines for "Care and use of animals in scientific research", established by Indian National Science Academy (INSA).

Subcutaneous tumours were grown by injecting 15×10^6 cells (in 0.1-0.15 ml volume) into the flank region of right hind leg. Tumour volume was calculated using the formula: \( V = \pi/6 \times d_1 \times d_2 \times d_3 \), where \( d_1 \), \( d_2 \) and \( d_3 \) are the three orthogonal diameters measured with the help of a calipers. Experiments were performed when the tumours had attained an average diameter of 1 ± 0.1 cm resulting in a volume of 0.5-0.6 cm^3 (6-7 days after implantation).

Administration of drugs — Tumour bearing mice were held in restrainers and H-342 as well as 2-DG was injected in the tail vein. The injection volumes of solutions of these chemicals prepared in normal saline were 0.1–0.15 ml. Unless mentioned otherwise, H-342 (0.5–10.0 mg/kg body wt) was always administered 1 hr before irradiation, while 2-DG (1–2 g/kg body wt) was administered immediately before irradiation.

Tumour irradiation and follow-up — Mice were held on a Styrofoam platform and restrained by adhesive tapes. Tumours were irradiated using 60Co teletherapy unit (Eldorado, AECL, Canada) by positioning them in a field size of 2 × 2 cm^2 achieved with the help of appropriate beam shapers at a tumour (sample) to source distance (ssd) of 80 cm. A total dose of 10 Gy was delivered at a dose rate of ~0.4 Gy/min. Ten to twenty animals were recruited into each group in these studies.

Tumour volumes were measured on alternate days. Animals with complete tumour regression were observed for their general condition including body weight till death.

Statistical analysis — Significance of differences in the mean values of tumour doubling time, \( t_d \), and percentages of cure rates were evaluated by standard statistical methods (Student's t test and Lawshe-Baker nomograph, respectively).

Results

Modification of radiation response of Ehrlich ascites tumour by H-342

Tumour growth delay — The initial growth of tumours in untreated controls was Gompertzian in nature. Administration of H-342 (0.5–10.0 mg/kg body wt) did not cause any significant effect on the tumour growth (Fig. 1). The average initial doubling times \( (t_d) \), were comparable to the values in untreated tumours (4.5-5.0 days) even at higher doses (5 and 10 mg/kg body wt) of H-342.

Irradiation of the tumour with Co-60 gamma-rays at an absorbed dose of 10 Gy (single fraction) induced only a transient delay in the growth (8-10 days; Fig. 1) with the mean value of \( t_d \) increasing to nearly 14 days. This absorbed dose, however, did not lead to a complete regression of the tumour in any of the irradiated mice (Table 1). Administration of H-342 1 hr before irradiation did not significantly alter the tumour growth up to a dose of 1 mg/kg body wt but delayed the growth in a dose dependent manner at
higher doses (Fig. 2). Complete tumour regression was observed only at the highest dose of H-342 used (10 mg/kg body wt) which induced nearly 55% of the tumours to regress completely.

Survival: Kaplan-Meier plots of actuarial survival are presented in Fig. 3. The median survival of untreated or sham irradiated mice bearing the tumour was 32 days from the day of treatment (1 week after implantation) and all the animals died within 40 days. Focal irradiation of the tumour (10 Gy) enhanced the survival by only 10 days, the median survival being 42 days (Table 1). Administration of H-342 before irradiation did not alter the survival up to a dose of 2 mg/kg body wt (data not shown). However, a dose of 5 mg/kg body wt marginally enhanced the survival (Fig. 3), the median survival being 50 days. Interestingly, increasing the dose to 10 mg/kg body wt resulted in a significant enhancement of the survival (median survival = 144 days).

Radiosensitization of EAT by a combination of H-342 and 2-DG

The growth of unirradiated tumours was not significantly altered by the administration of 2-DG (2 g/kg body wt) alone or in combination with H-342.

Administration of 2-DG (2 g/kg body wt) just before irradiation delayed significantly the growth of the tumour (Fig. 1) as compared to radiation alone, the mean value of $t_d$ being 45 days. 2-DG in combination with radiation also induced complete tumour regressions in nearly 45% of the mice ($P<0.001$; Table 1). Administration of H-342 along with 2-DG before irradiation, did not significantly alter the growth de-

<table>
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<th>Gammas-rays (Gy)</th>
<th>H-342 (mg/kg body wt)</th>
<th>2-DG (g/kg body wt)</th>
<th>No. of animals (n)</th>
<th>Tumour doubling time ( (t_d) ) (days)</th>
<th>Median survival time (days)</th>
<th>Cure ( (%) )</th>
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\(^a\) Cure is defined as tumour free survival for more than 100 days

\(^b\) Significantly different than unirradiated tumour \( (P<0.001) \)

\(^c\) Significantly different than irradiated tumour \( (P<0.001) \)

\(^d\) Significantly different than irradiated tumour treated with 2-DG \( (P<0.05) \)
lay or the median survival time of mice any further. Interestingly however, the combination at a H-342 dose of 5 mg/kg body weight enhanced the cure rate to 66% as against 45% (P<0.05) observed in mice treated with 2-DG plus radiation, whereas, at a higher dose of H-342 (10 mg/kg body wt) additive effects due to 2-DG were not observed (Table 1).

Discussion

Contrary to expectations, the results of the present studies clearly demonstrate the radiosensitizing effects of the DNA ligand H-342 in the Ehrlich ascites tumour irradiated in vivo. Present studies have also shown that the combination of H-342 (5 mg/kg body wt) with the glucose analogue, 2-DG could be more effective in enhancing the tumour cure rate (Table 1). Since the combination (H-342 + 2-DG) has been earlier shown to effectively protect normal tissues (like the bone marrow) against radiation damage31, therefore, this combination of radiomodifiers is expected to produce a large differential effect between the normal and tumour tissues. Present results could have important implications for optimizing tumour radiotherapy, since the doses at which these agents demonstrate the tumour radiosensitizing effects are neither toxic nor mutagenic42. The two approaches used here for radiosensitization, namely modification of energy flow (using 2-DG) and information processing (using H-342) provided cure rates (tumor free survival of >100 days) of more than 60% in combination with radiation. The fact, that the animals after therapy survived for more than a year indicates the absence of any undesirable side effects.

The delay in tumour growth induced by H-342 (Figs. 1 & 2) and H-342+2-DG in combination with radiation, could arise on account of cytotstatic as well as cytotoxic effects of these treatments. Both H-342 and 2-DG are known to induce concentration dependent division delay in un-irradiated as well as irradiated cells in vitro,33,34. However, both these agents (alone or in combination) did not induce any significant growth delay in unirradiated tumours (Table 1).

A combination of gamma irradiation with H-342 at a higher dose (10 mg/kg body wt) resulted in a complete regression of the tumour in 55% of the animals. However, together with 2-DG, the combination at this dose did not significantly increase either the median survival or the cure rate, although an increase in t_d was observed. The data on tumour doubling time do not correlate strictly with survival or the cure rate (Table 1). Since disease free survival is the ultimate aim of any cancer treatment, careful and ethically permissible observations on animal survival in experimental oncology studies are considered essential to develop and evaluate new therapeutic approaches. Therefore, animals showing complete tumour regression are being followed till death.

The mechanisms underlying radiomodifying action of H-342 are complex and as yet not clearly understood. However, their ability to scavenge OH radicals as well as quench DNA radicals could be important as shown in simple in vitro systems,26,27,35,36. In vivo, H-342 and H-258 could further interfere in the catalytic action of topoisomerases, important enzymes involved in a number of DNA transactions including DNA repair,37,38. Camptothecin and etoposide, inhibitors of topoisomerase I and II, have been shown to potentiate the cytotoxic effects of radiation.39
Binding of these ligands could also alter the degree of chromatin condensation 40, which may modify the repair as well as fixation of DNA lesions. Since these processes are highly dependent on the concentration of H-342, dose dependent modifications in tumour radioreponse are likely.

Further, it has also been demonstrated that the dissociation of DNA bound Hoechst and topoisomerase activities (particularly topo II) are energy dependent 41,42. Available data support the hypothesis that the effects of the glucose antimetabolite, 2-DG, could be mediated through the energy linked modification of the repair and fixation of radiation-induced lesions 33,16. Therefore, it appears that the radiomodifying actions of H-342 and 2-DG could be interrelated and may involve some common sites and pathways.

An important consideration, while using agents that interact with DNA, is mutagenicity, besides any acute toxicity. Induction of 6-thioguanine resistant mutants by H-342 have been demonstrated in cell cultures (V 79 cells), albeit at high concentrations (> 50 μM) 33. The maximum dose of H-342 that demonstrated its radiosensitizing effects (10 mg/kg body wt) is considerably below the LD50 dose in mice (300 mg/kg body wt) 33. Preliminary experiments on the induction of cytogenetic damage in bone marrow cells of mice indicate that, even the higher doses of H-342 (5 and 10 mg/kg body wt) used in the present work induced only a transient increase in the micronuclei formation from 0.2% (controls) to 0.5% and 0.8%, respectively, which is not very alarming.

In conclusion, present studies suggest that the combination of H-342 and 2-DG could be useful in improving the efficacy of tumour radiotherapy. Therefore, further preclinical investigations and studies to understand the underlying mechanisms responsible for the tumour radiosensitizing effects of the combination of H-342 with 2-DG are warranted.

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