Radiosensitization of a mouse melanoma by withaferin A: *In vivo* studies

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The radiosensitizing effect of a plant withanolide, withaferin A, on the B16Fl mouse melanoma was studied *in vivo*. Treatment of 100 mm³ tumours with 10 to 60 mg/kg withaferin A intraperitoneally produced a dose dependent increase in growth delay and volume doubling time. Injection of 30-50 mg/kg withaferin A, followed by 30 Gy local gamma irradiation, significantly enhanced the tumour response. No systemic or local adverse reactions were noted in these groups. The drug was most effective when injected intraperitoneally 1h before irradiation. However, neither the individual agents nor their combination could produce any complete response (tumour cure). Melanoma is a relatively radioresistant tumour. The present results indicate that the radiation response of this tumour can be significantly enhanced by pretreatment with withaferin A.

Withaferin A (4β, 27-dihydroxy-1-oxo 5β, 6β, epoxy witha 2-24 dienolid e), a steroidal lactone, is found in the leaves and roots of the Indian medicinal plant, *Withania somnifera*. Withaferin A has been demonstrated to have cytotoxic as well as radiosensitizing effects on mouse Ehrlich ascites carcinoma *in vivo* and Chinese hamster V79 cells *in vitro*. In these studies the compound was introduced directly into the tumour (intraperitoneally) or the culture medium, which may not be feasible in most human cancers. Solid tumours are good models for testing new anticancer drugs and chemical radiosensitizers. A recent study showed that administration of WA at 30-50 mg/kg b.wt. before a local irradiation significantly increased the cure rate of a radioresponsive tumour mouse fibrosarcoma, *in vivo*. The presence of radioresistant hypoxic cells is the major obstacle in solid tumour radiotherapy. Therefore, agents which can specifically act on such tumors to increase radiosensitivity can improve therapeutic outcome. B16Fl mouse melanoma is a radioresistant tumour. Therefore, this tumour was chosen as a model in this study to determine the cytotoxic and radiosensitizing effects of withaferin A on radioresistant tumours.

Materials and Methods

Tumour—B16Fl mouse melanoma was originally procured from the Cancer Research Institute, Mumbai, India, and propagated by serial transplantation on the dorsal skin of adult C57BL mice of either sex. For experiments, 5×10⁵ viable cells were injected intradermally on the dorsal skin. Once the palpable tumour developed, its diameters in three perpendicular planes (D₁, D₂, D₃) were measured using a vernier caliper and the tumour volume (V) was calculated using the formula:

\[ V = \frac{1}{6} D_1 D_2 D_3. \]

Tumours measuring 100 ± 10 mm³ were used for the experiments.

Drug—*Withania somnifera* root powder was a kind gift from Vaipa Pharmaceuticals Ltd. Gujarat, India. Ethanolic extract of the root powder was prepared by the method of Suffness and Douros and withaferin A (WA) was isolated by the method of Subramanian and Sethi as described earlier. WA was dissolved in a few drops of ethanol and a homogeneous suspension was made with 0.5% carboxy methyl cellulose (CMC) in phosphate buffered saline, freshly before each experiment.

Irradiation (RT)—Animals were anaesthetized by intraperitoneal (i.p.) injection of Ketamine (50 mg/kg, Themis, Bombay, India) and diazepam (0.5 mg/mouse, Ranbaxy, New Delhi, India). Local irradiation of the tumour was done with a ¹⁰³Co teletherapy source (Siemens, Germany) at a dose rate of 1 Gy/min, in a field size of 4 × 4 cm.
**Experimental design**

The experiments were conducted according to our institutional regulations and national criteria for animal experiments.

The following experiments were carried out:

**Route of administration**—To select the best route of administration, 40 or 60 mg/kg body wt. of WA was given i.p., i.m. or p.o. to tumour-bearing mice, using 10 animals in each group.

**Time of drug administration with RT**—Groups of 10 tumour-bearing mice were injected i.p. with 40 mg/kg WA, at 30 min, 1 hr, 2 hr, 4 hr, 8 hr or 12 hr before or 30 min, 1 hr or 2 hr after local exposure to 30 Gy. The radiation dose was selected on the basis of a preliminary study using 10 Gy to 50 Gy of radiation with 40 mg/kg of WA.

**WA dose response**—Different groups of ten tumour-bearing mice were injected i.p. with 10, 20, 30, 40, 50 or 60 mg/kg of WA alone or followed by a local irradiation of tumour with 30 Gy, 1 hr after drug injection. One group of ten animals was injected i.p. with 0.2 ml of vehicle (CMC) and then locally irradiated 1 h later, as above.

**Parameters**

The animals were observed for 120 days or till dead. The following parameters were studied:

- volume doubling time (VDT): time required for the tumour to attain double the treatment volume;
- growth delay (GD): the difference in time, in days, between the treated and the untreated tumours to reach 5 times the treatment volume;
- complete response (CR): complete regression with no regrowth at the primary site during the 120 days of observation;
- partial response (PR): a regression of 50% or more from the treatment volume;
- no response (NR): less than 50% regression in the treatment volume.

**Statistical evaluation**

Statistical evaluation was done using Student's t-test. Dose response curves were drawn by fitting the data to linear (Y = C + αD) model using LOTUS 1-2-3 software.

**Results**

**Route of administration**—Out of the three routes used, i.p. gave the best results at 40 mg/kg. At this dose, i.p. administration produced significantly ($P < 0.001$) higher VDT and GD compared to the other routes. The tumour response by i.p. injection was not markedly affected by increasing WA dose from 40 to 60 mg/kg. Oral administration of 60 mg/kg WA significantly increased the VDT and GD above that produced by 40 mg/kg p.o., and these values were
comparable to those after i.p. administration. The effect of i.m. administration was also significantly (VDT, $P < 0.05$, GD, $P < 0.001$) enhanced by increasing the drug dose from 40 to 60 mg/kg, but the effect was less pronounced than with oral administration (Fig.1).

**Time of administration with RT**—Based on the above results, 40 mg/kg of WA i.p. was selected for studying the effect of different time intervals between drug and RT on tumour response. Administration of WA 1 h before RT (30 Gy) was the most effective, giving significantly higher GD and VDT ($P < 0.001$) compared to RT alone. A significant increase in the values could be obtained even when WA was given 30 min before RT. The effect decreased with increase in the interval beyond 1 h between WA injection and irradiation, although a higher GD was evident also in the groups given WA 2 hr before and 30 min after RT (Fig. 2).

**WA dose response**—No complete or partial response was obtained even at the highest dose of WA used in this study. Doses from 10 to 60 mg/kg produced a linear dose-dependent increase in both VDT ($r^2 = 0.947$) and GD ($r^2 = 0.937$). The VDT became significantly higher than control only at 30 mg/kg and above ($P < 0.01$-$0.001$). The values showed a significant increase with each dose increment of 10 mg/kg from 20 to 40 mg/kg. Further increase in dose did not produce a proportional increase in effect. A similar response was seen for GD with dose increments above 10 mg/kg. The maximum GD and VDT obtained with WA, as an individual modality, was less than 6 days (Table I, Fig. 3). With the addition of one dose of 30 Gy with WA treatment, both GD and VDT increased significantly above that of WA alone. But the increase in VDT became significant compared to the RT alone group only from 30 mg/kg, while increase in GD was significant even at 20 mg/kg. Increase in WA dose from 30 to 50 mg/kg with RT resulted in significant increases in both VDT and GD. No systemic or local side effects were noticed in these groups. Increase in WA dose above 50 mg/kg did not

| Table 1—Changes in VDT and GD after treatment with different doses of WA |
|-----------------------------|-----------------|----------------|
| Dose of WA (mg/kg)         | VDT (Days ± SEM) | GD (Days ± SEM) |
| 0 (Control)                | 3.50 ± 0.29     | —              |
| 10                         | 3.72 ± 0.16     | 0.89 ± 0.34    |
| 20                         | 3.97 ± 0.14     | 1.89 ± 0.29*   |
| 30                         | 4.87 ± 0.19b    | 2.71 ± 0.89*   |
| 40                         | 5.50 ± 0.14c    | 4.13 ± 0.28c   |
| 50                         | 5.70 ± 0.11c    | 4.38 ± 0.36    |
| 60                         | 5.83 ± 0.11c    | 4.41 ± 0.36a   |

P values: $^a < 0.05$; $^b < 0.05$ compared to control
$^c < 0.01$; $^d < 0.001$ compared to 20 mg/kg
$^a < 0.01$; $^e < 0.001$ compared to 30 mg/kg
$^* < 0.05$ compared to 10 mg/kg

Fig. 2.—Effect of changes in time interval between WA injection and irradiation on tumour response

$P$ values $^c < 0.05$; $^d < 0.01$; $^e < 0.001$ compared to RT; ■ - VDT, □ - GD
produce any marked advantage over the immediate lower dose (Table 2, Fig. 4). As in the case of WA alone, the drug dose response was linear when combined with RT also (VDT: $r^2 = 0.934$, GD: $r^2 = 0.994$).

**Discussion**

The present study has demonstrated that i.p. injection produced the best results at 40 mg/kg of WA and this effect is not increased by increasing drug dose. This agrees with the earlier findings that 30 mg/kg was the optimum dose, where only i.p. route was tested\(^3\), \(^4\). However, the present results also show that the effectiveness of the oral route can be increased by using a higher dose (60 mg/kg) of WA. This is of significance in the clinical application of the drug, as oral route will be preferable if the drug is to be administered daily with conventional radiotherapy. The toxicity was also lower after oral administration than after i.p. injection, as reported in another study\(^5\).

When injected i.p., the maximum tumour response was obtained with an 1 hr interval between WA and
RT and the effect decreased with the decrease or increase in this interval. The finding that pretreatment with WA gives significantly higher radiosensitization than post-treatment suggests that the drug must be interacting with the critical target molecules so as to make them more susceptible to radiation. WA has been reported to arrest cells in mitosis, which will result in synchronization of cells in a more radiosensitive phase. An in vitro study has shown that WA enhanced the Fenton reaction-generated free radical activity in vitro, which reached a peak at 1 hr after addition of the drug. Such a reaction at a time when cells are in a radioresponsive phase can produce a higher cell killing, resulting in the enhanced tumour response seen in the present study.

Although the optimum interval between WA and RT appears to be 1 hr, the finding that some modification is obtained even when the drug is given 2 hr before irradiation, suggests that WA is not metabolized very fast. However, this aspect needs to be investigated. A similar potentiating effect observed even when WA was given 30 min after RT may be due to a repair inhibitory action of the drug. Fuska et al. have shown that WA inhibited DNA and protein syntheses in P388 cells, while Chaudhary and Neogy reported inhibition of RNA and protein syntheses in mouse tumours by this drug. Flow cytometric studies in V79 cells have indicated that WA interferes with cell cycle. Inhibition of synthesis of macromolecules essential for DNA repair will result in inhibition of damage repair leading to cell death.

The present finding that WA enhances the radioresponse of mouse melanoma and that the effect increases linearly with drug dose supports our earlier findings on exponentially growing Ehrlich ascites carcinoma (EAC) and on mouse fibrosarcoma. In the latter study also it was observed that increase in WA dose above 40 mg/kg did not produce a proportionate increase in tumour free survival, which is similar to the present observation on melanoma. Unlike in the EAC bearing Swiss mice, where doses above 30 mg/kg were not tolerated with radiation, in the melanoma even 50 mg/kg WA given before RT (30 Gy) did not produce any toxic side effects and significantly enhanced the tumour response compared to 30 or 40 mg/kg + RT. The higher toxicity in the case of EAC could be explained by the intraperitoneal location of the tumour, where the small intestine, which is a radiation dose limiting normal tissue, is fully exposed to the treatment. But in the present investigation, none of the WA+RT treated animals showed any notable skin reaction at the tumour site or systemic toxicity with the optimum drug dose. Therefore, WA appears to be better suited for sensitization of solid tumours exposed locally, where the involvement of critical normal tissues like small intestine is minimal. This study indicates that WA may have promise as an adjuvant to radiotherapy for increasing the therapeutic response of relatively resistant solid tumours. The exact mechanism of radiosensitization by WA is not known. However, inhibition of repair of radiation induced damage and depletion of cellular antioxidants are likely to contribute to the radiosensitizing action of WA. Ashwagandha root extract, which is the source of WA, has been shown to decrease the glutathione content in mouse Sarcoma-180 in vivo. A study is planned to see if WA has a similar effect on the mouse melanoma.

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