Acta Oncologica Vol. 35, No. 1, pp. 95–100, 1996

ANTITUMOR AND RADIOSENSITIZING EFFECTS OF WITHAFERIN A ON MOUSE EHRLICH ASCITES CARCINOMA IN VIVO

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The antitumor and radiosensitizing effects of withaferin A (WA), a steroidal lactone from Withania somnifera, was studied on Ehrlich ascites carcinoma in vivo. The acute $LD_{50(14)}$ for WA in Swiss mice was ~80 mg/kg. Twenty-four hours after i.p. inoculation of 10^6 tumor cells, WA was injected i.p. at different dose fractions (5 or 7.5 mg/kg × 8, 10 mg/kg × 5, 20 or 30 mg/kg × 2) with or without abdominal gamma irradiation (RT, 7.5 Gy) after the first drug dose. Increase in life span and tumor-free survival were studied up to 120 days. The drug inhibited tumor growth and increased survival, which was dependent on the WA dose per fraction rather than the total dose. Combination of RT with all the drug schedules increased tumor cure and tumor-free survival, the best effect seen after 2 fractions of 30 mg/kg each. In another experiment WA was given as 2 (40 mg/kg × 2), 3 (30 mg/kg × 3) or 4 (20 mg/kg × 4) fractions at 5, 7 or 10 days after tumor inoculation with or without RT after the first drug dose. At 7 and 10 days after inoculation the drug was effective only at 40 mg/kg × 2, but with RT 30 mg/kg × 3 produced an equal effect (20% survival) on 7 day old tumors.

Ayurveda, the Indian traditional system of medicine, contains many herbal remedies thought to be active against a variety of diseases, including cancer. These drugs have not been explored to any great extent and systematic search into Ayurveda medicine may yield valuable information for the development of new anticancer and/or radiosensitizing drugs. A good example of anticancer drug development for other plant products is that of the vinca alkaloids.

Withania somnifera, popularly known as Ashwagandha, is a medicinal plant, which has a vast range of therapeutic applications (1–3). Earlier authors have attributed the pharmacological and therapeutic properties of Ashwagandha to the withanolides in the extract, especially withaferin A (4). Kupchan et al. (5) and Lavie et al. (6) showed that withaferin A had marked tumor inhibitory activity when tested in vitro against cells derived from human nasopharyngeal carcinoma, while Shohat et al. (7, 8) and Shohat & Joshua (9) found antitumor and immunostimulating effects in tumor-bearing mice.

Preliminary studies in our laboratory have shown that the ethanolic extract of Ashwagandha roots is very effective against mouse Sarcoma-180 solid tumor in vivo (10) and that the tumor killing effect can be significantly increased by combining Ashwagandha treatment with irradiation and hyperthermia (11). Moreover, the extract showed no apparent toxicity on any of the systems (12). The crude extract was also effective in prolonging the life span of mice bearing Ehrlich ascites tumor (13). These encouraging results prompted us to evaluate the antitumor and radiosensitizing effects of the purified component withaferin A in mouse Ehrlich ascites carcinoma.

Received 21 February 1994.
Accepted 16 December 1994.
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 ISSN 0284-186X

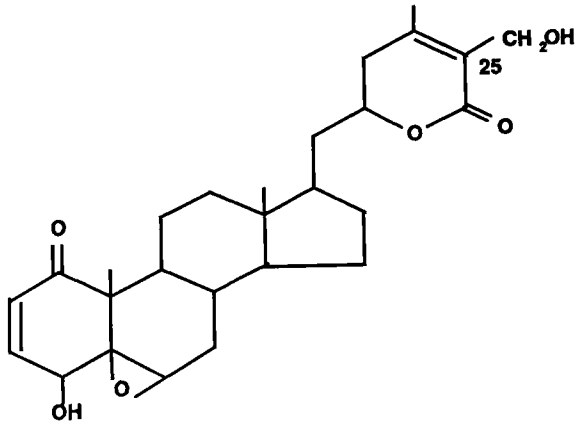

Fig. 1. Chemical structure of withaferin A (4β,27-dihydroxy-1-oxo 5β,6β, epoxy witha 2–24 dienolide).

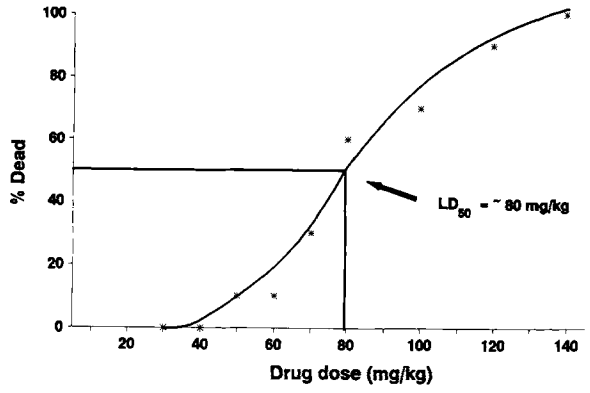

Fig. 2. Fourteen-day mortality data plotted as percent dead versus drug dose. The LD_{50} of the drug = ~ 80 mg/kg body wt.

Material and Methods

Animals and tumor model. Inbred Swiss albino mice of either sex, 6–8 weeks of age and weighing 25–30 g, were used. They were maintained under controlled temperature and humidity, with sterile bedding and standard mouse food and water ad libitum. Ehrlich ascites tumor was maintained and propagated by serial transplantation intraperitoneally in adult female mice. This tumor cell line has not shown any spontaneous regression in our mouse colony. For experiments, 10^6 tumor cells were inoculated i.p.

Drug. Withania somnifera root powder (Vaipa Pharmaceuticals Ltd., Gujarat, India) was purchased locally and the alcoholic extract was prepared by the method of Suffness & Douros (14). The solvent was evaporated in a Speedvac vacuum dryer (Savant, USA). One kilogram root powder yielded 100 g extract. Withaferin A was isolated from the alcoholic extract by fractionation and chromatographic separation as described by Subramanian & Sethi (4). The final product is a greenish yellow crystalline substance identified as withaferin A (WA). The yield of WA may vary from 0.01–0.1% depending on the time of collection, area of cultivation and method of extraction. In our sample the yield was ~0.1% of root powder. The purity of the sample was confirmed by color reactions, co-chromatography and UV spectra compared with authentic sample obtained from Dr. P. D. Sethi, Director, Central Indian Pharmacopoeia Laboratory, Ghaziabad, India. WA has a molecular weight of 470. The structure is

Table 1

Effect of Withaferin A (WA) and irradiation (RT) on the Ehrlichs ascites tumor bearing mice

Treatment #	Schedule Day(s)	Median survival time (days)	Increase of MST (%)	Av. weight * change (%)	Survivors (%) on day 120	Synergy **
1. Control (saline)	1–8	19	0	+5.1	0	
2. WA-5	1–8	22	16	−8.3	20	
WA-7.5	1–8	26 [a]	37	−8.5	20	
WA-10	1–5	29 [c]	53	−8.0	50	
WA-20	1,3	48 [c]	153	−1.8	70	
WA-30	1,5	72 [c]	279	−6.2	80	
3. RT(7.5Gy)	1	26 [a]	37	−5.2	0	
4. WA-5 + RT	1–8	27 [b]	42	−8.9	40	2
WA-7.5 + RT	1–8	33 [c]	74	−9.5	60	3
WA-10 + RT	1–5	29 [c]	53	−12.6	80	1.6
WA-20 + RT	1,3	28 [c]	47	−8.8	90	1.28
WA-30 + RT	1,5	-	-	−7.5	100	1.25

WA-5 to 30 indicate drug dose in mg/kg.

* Determined at 1 week of treatment.

a,b,c—significant compared to control a = $p < 0.05$, b = $p < 0.005$; c = $p < 0.0001$.

** synergy-factor > 1 indicates synergy.

Table 2

Influence of tumor size on the antitumor effect of Withaferin A (WA) and irradiation (RT) on mice bearing Ehrlich ascites tumor.

Treatment #	Schedule day(s)	Median survival time (days)	Increase of MST (%)	Survivors (%) on day 120	S-factor **
1. Control (saline)	5, 7, 10, 12	19		–	
2. WA-20	5, 7, 10, 12	26 [a]	37	40	
WA-30	5, 7, 10, 12	32 [b]	68	60	
WA-30	7, 10, 12	21	11	–	
WA-40	10, 12	20.5	8	20	
3. RT(7.5Gy)	5	26 [a]	37	–	
	7	28 [b]	47	–	
	10	19	–	Inactive	
4. WA-20 + RT	5, 7, 10, 12	30.5 [b]	61	60	1.5
WA-30 + RT	5, 7, 10	40 [c]	111	80	1.3
WA-30 + RT	7, 10, 12	26.5 [b]	39	20	
WA-40 + RT	10, 12	15		Toxic	

#WA-20 to 40 indicate drug doses in mg/kg.
a, b, c,—significant compared to control.
[a = $p < 0.05$, b = $p < 0.005$] c = $p < 0.0001$]
**synergy factor > 1 indicates synergy.

given in Fig. 1. The drug was prepared freshly before use. It is not soluble in water. It was dissolved in a few drops of ethanol and a homogeneous suspension was made with normal saline containing 0.5% carboxymethyl cellulose (CMC).

Irradiation. Irradiation was done with a ^{60}Co gamma-tron teletherapy unit (Siemens, Germany) at a dose rate of 0.75 Gy/min under ketamine (50 mg/kg body wt.) anaesthesia.

Toxicity profile. The acute intraperitoneal (i.p.) toxicity of WA was evaluated in albino mice as described by Ghosh (15). The animals were fasted overnight (18 h). Graded doses of WA, 30, 40, 50, 60, 70, 80, 100, 120 or 140 mg/kg, were administered i.p. to different groups of 10 mice each. The animals were observed for a period of 14 days for drug induced mortality. Percent mortality was plotted against drug dose. The LD_{50} of the drug was calculated from the graph (Fig. 2).

Tumor take and growth. TD_{50} was determined by injecting i.p. different number of cells into groups of 10 mice each and following tumor growth. TD_{50} was calculated by plotting the percent tumor take within one month against the number of cells injected.

Tumor growth inhibition. Twenty-four hours after tumor cell injection the animals were divided into groups with 10 animals in each and treated as follows:

1) Control: 0.2 ml of saline containing 0.5% carboxy methyl cellulose for 10 consecutive days.
2) WA: 5, 7.5, 10, 20 or 30 mg/kg was injected i.p. once daily as indicated in Table 1.
3) Irradiation (RT) only: one dose of 7.5 Gy locally to the abdomen on day 1.
4) WA + RT: 7.5 Gy abdominal irradiation within 1 h after the first dose of WA.

Influence of tumor size on the antitumor effect of WA and radiation. Tumors were allowed to grow for 5, 7, or 10 days and treated with different drug dose fractions and schedules, with or without irradiation, as shown in Table 2.

All the mice were weighed on the day of tumor inoculation and at weekly intervals. Animal survival was recorded up to 120 days. The survival of animals up to 120 days is roughly equivalent to 5 year survival in man (16). The tumor response was assessed on the basis of median survival time and tumor-free survival. Median survival time (MST) was calculated from the animals dying within 120 days and those surviving 120 days were excluded from these calculations.

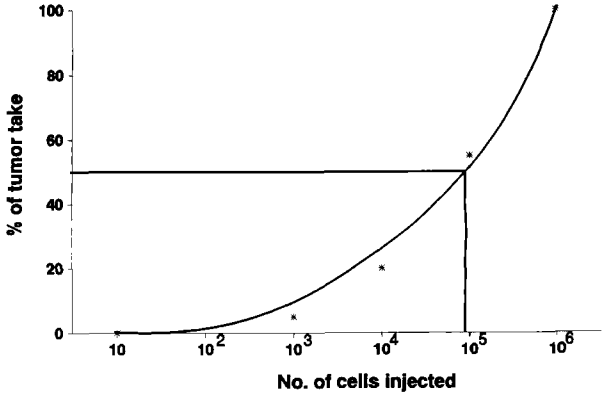

Fig. 3. Percent tumor take as a function of the number of tumor cells injected. The $TD_{50} = \sim 10^5$ cells.

Statistical analysis. Statistical evaluation of the data was done by Student's t-test. A value of $p < 0.05$ was considered significant. The interaction of drug and radiation was assessed by the S-factor (index of synergy), calculated according to Rothman (17) from the expected probability (P_{exp}) and the observed probability (P_{obs}) of the effect:

$$S = \frac{P_{obs}}{P_{exp}} \text{ where } P_{exp} = P_A + P_B$$

P_A and P_B are the probabilities of an effect after treatment with agent A and agent B.

Results

Acute toxicity. No death was observed within 14 days after a single i.p. injection of 30 or 40 mg/kg of WA, and even up to 60 mg/kg the mortality was marginal. There was a sharp increase in mortality above 70 mg/kg of the drug. Doses of 100 mg/kg and above produced severe toxic symptoms like ruffling of hair, diarrhea and severe weight loss. The LD_{50} was found to be ~ 80 mg/kg body wt. (Fig. 2).

Tumor take and growth. The TD_{50} was found to be $\sim 10^5$ cells (Fig. 3). All the animals injected with 10^6 cells developed tumor and died within 20 days. No spontaneous regression of tumor was seen.

Tumor growth inhibition. WA at 5 mg/kg (total dose = 40 mg/kg) and 7.5 mg/kg (total dose of 60 mg/kg) for 8 consecutive days produced 20% tumor cure and all animals survived > 120 days. Increasing the dose per fraction to 10 mg/kg (total dose = 50 mg/kg) for 5 days increased both MST and tumor-free survival, which was independent of the total dose administered. Further increase in the dose per fraction to 20 mg/kg with a reduction in the number of fractions to 2 (total dose = 40 mg/kg) increased the MST to 48 days and tumor-free survivors to 70%. Two doses of 30 mg/kg per fraction (total dose = 60 mg/kg) gave 72 days of MST, but survival increased by only 10% above that produced by 2 fractions of 20 mg/kg. Irradiation alone did not produce any tumor-free survivors, though there was an increase in the life span comparable to that produced by 8 days treatment with 7.5 mg/kg WA. Combination of RT with all the drug schedules resulted in an increase of the tumor-free survival rate compared to the respective drug treatments alone. Eight doses of 5 mg/kg (40 mg/kg) of WA with RT gave 40% tumor-free survivors. An increase in dose per fraction to 7.5 mg/kg for 8 days (60 mg/kg) or 10 mg/kg for 5 days (50 mg/kg) with RT further enhanced the tumor-free survival to 60 and 80% respectively. With 20 mg/kg WA × 2 days, the combination gave 90% survival at 120 days, while 30 mg/kg WA × 2 with radiation treatment gave a 120-day survival of 100% (Table 1). The drug treatment caused a reduction in the animal weight during the course of treatment, but all the surviving animals regained normal weight within a week of cessation of treatment. None of these animals exhibited any late toxicity symptoms.

Influence of tumor size on the antitumor effect of WA. Four doses of 20 mg/kg on days 5, 7, 10 and 12 (total dose of 80 mg/kg) after tumor cell inoculation gave a MST of 26 days with 40% tumor-free survival at 120 days. Increase of dose to 30 mg/kg × 3 (total dose = 90 mg/kg) on days 5, 7 and 10 enhanced the MST to 32 days with a 20% increase in 120-day survival above that observed after the low-dose fractions. However, the same schedule in advanced tumor stages (days 7, 10 and 12) was ineffective. Two doses of 40 mg/kg on days 10 and 12 (total dose = 80 mg/kg) resulted in 20% tumor cure but did not increase MST of the uncured animals above that of the controls (Table 2). The effect of irradiation decreased as the tumor size increased and irradiation seemed ineffective in advanced tumors. A single dose of 7.5 Gy on days 5 or 7 resulted in an MST of 26 and 28 days (7 and 9 days above control) respectively. However, it failed to produce any long-term survival. Irradiation of 10-day tumors did not have any beneficial effect (Table 2).

Combination of RT with all the drug schedules resulted in an increase in tumor-free survivors compared to the respective drug alone treatments. Combination with one dose of RT with 4 doses of 20 mg/kg (on days 5, 7, 10 and 12, total dose = 80 mg/kg) of WA increased the MST to 30 days ($p < 0.005$) and tumor-free survival at 120 days to 60%. An increase in dose per fraction to 30 mg/kg with a reduction in number of fractions to three (on days 5, 7 and 10, total dose = 90 mg/kg) + RT increased tumor-free survivors to 80%. The same schedule also produced a significant ($p < 0.05$) increase in MST (21 days above that of control) as well as tumor-free survival compared to the individual modality treatments, even when the treatments were given at more advanced stages (7, 10 and 12 days) of tumor growth. Higher drug dose of 40 mg/kg on days 10 and 12 (total dose 80 mg/kg) in combination with RT was toxic and the animals died before any antitumor effect could be assessed (Table 2).

Discussion

The acute LD_{50} at 14 days (~ 80 mg/kg) of the present study was not markedly different from the LD_{50} at 72 h (87 mg/kg (18)). The LD_{50} in the present study was higher than that reported by Shohat et al. (8) for the drug from an Israeli plant (54 mg/kg body wt.). Indian plants differ from the known chemotypes of the Israeli plant in the composition of withanolides and the differences in chemical composition also depend on the variations in species, environment and methods of isolation (1).

A good response of Ehrlich ascites tumor to withaferin A isolated from the leaves of the Israeli variety of W. somnifera has been reported earlier by Shohat et al. (8),

while Das et al. (19) observed that withanolide D, another steroidal lactone occurring in the leaves of W. somnifera Dunal, possesses significant activity against Sarcoma-180 and Ehrlich ascites carcinoma. The present results confirm the antitumor effect and also demonstrate a radiosensitizing potential of withaferin A.

The present results on 24-h-old tumors indicate that the drug dose per fraction may be more important than the total dose of the drug. This suggests that the tumor killing effect may not be cumulative and that the tumor cells may be able to recover from the small toxic insults induced by the low doses during the interval between fractions. As the drug is injected directly into the peritoneal cavity where the tumor is growing, the effect would be immediate and direct on the tumor cells. Palyi et al. (20) reported that WA arrested mitosis in HeLa cells in culture, while Shohat et al. (8) and Chowdhury & Neogy (21) observed inhibition of RNA synthesis in Sarcoma-180 ascites tumor cells. Kupchan et al. (22) reported that sesquiterpene lactones, possessing lactone and epoxide rings like that of WA, inhibit the SH-groups of enzymes. An inhibition of protein and nucleic acid synthesis following treatment with WA and its derivatives was found by Fuska et al. (23) in P388 cells in vitro. This may explain the potent cytotoxic properties of withaferin A.

In combination with irradiation on first day after tumor inoculation, the drug at all fraction schedules yielded better results than those produced by RT or drug alone; there was a synergistic increase of tumor-free survival. Moreover, the increase in effect with combination treatment was more pronounced at lower drug dose fractions than at higher fractions.

Even though RT increased the MST when treated on 1–7 days, it was ineffective in prolonging survival when the treatment was given to 10-day-old tumors. This may be due to the proliferation status of the tumor. The tumor growth is exponential during the first 7–8 days, but after that it becomes stationary and on the 10th day a large fraction of the cells are in G_1 phase (24), which is radioresistant.

Even the advanced tumors responded to the WA + RT combination treatment, which produced a synergistic increase of the 120-day survival. However, the maximum dose per fraction which could be used safely was 30 mg/kg for 3 days (total dose = 90 mg/kg). Increase in drug dose per fraction to 40 mg/kg (2 fractions) or increase in the number of fractions at 30 mg/kg to > 3 with RT was not tolerated. From the present results, higher dose per fraction (40 mg/kg on day 10 and 12) appears to be effective for inducing tumor control in advanced tumor stage when used as single modality, with a possibility to reduce the drug dose to 30 mg/kg or 20 mg/kg when combined with irradiation. This suggests that withaferin A may be used as an adjuvant to increase the effect of radiotherapy. Cytotoxic and radiosensitizing effects of WA were also observed on Chinese hamster V79 cells in vitro (Uma Devi et al. under publication).

The present study showed that a total dose of 40 mg/kg ($\sim$ 1 mg/mouse) of withaferin A administered in two equal doses of 20 mg/kg each gave almost similar tumor cure and survival as 500 mg/kg $\times$ 10 of Ashwagandha extract + RT: Withaferin A, 50 mg/kg (10 mg/kg $\times$ 5) + RT produced almost identical effect as 750 mg/kg $\times$ 10 of Ashwagandha extract + RT (11). This indicates that withaferin A is the principal component of the extract responsible for the tumor inhibiting effect of the root extract. This is of significance, as the root powder of the plant is used in the crude form in the Indian system of medicine (1). Moreover, the crude extract (100 mg/kg $\times$ 30) was well tolerated and has an anabolic effect on mice, as evident from the increase in blood hemoglobin levels and body weight (12).

It can be concluded from the present study that withaferin A is effective in inhibiting the growth of Ehrlich ascites tumor in mouse. A small number of high dose fractions was more effective than a large number of smaller dose fractions. Even on advanced tumors the drug could exert good antitumor and radiomodifying effects. An important finding of the present study was the higher in vivo tumor killing when WA treatment was combined with irradiation and the synergistic effect of WA and irradiation. This phenomenon deserves further investigations.

ACKNOWLEDGMENTS

Senior Research Fellowships (SRF) to ACS from Council of Scientific & Industrial Research (CSIR) and to FES from Indian Council of Medical Research (ICMR), Govt. of India, are gratefully acknowledged. The kind gift of standard sample of withaferin A from Dr. P. D. Sethi, Director of Central Indian Pharmacopoeia Laboratory, Ghaziabad India, is thankfully acknowledged. Radiation facility was provided by Prof. K. Koteshwar Rao, Head, Department of Radiotherapy and Oncology, Kasturba Hospital, Manipal.

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