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Nafoxidine Possesses Antitumor Activity against an Ascitic Hepatoma

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Abstract—*Nafoxidine an antiestrogen is shown to possess antitumor activity against the Zajdela ascitic hepatoma. Nafoxidine prolonged the survival time of the rats injected with the tumor cells; complete regression of the tumor was observed when rats bearing a* 3-day-old *tumor were injected with this compound. Nafoxidine (Naf) also showed cytotoxic effects against the hepatoma cells* in vitro. *Naf competed with the binding of labelled estradiol to the tumor cells.*

INTRODUCTION

THE presence of estrogen receptors in liver cells and the role of steroids in liver-cell metabolism is well known [1]. Estradiol receptors have been also shown to be present in human and animal hepatomas [2]. Antiestrogens inhibit the action of estrogens by preventing the binding of estradiol to cytosol receptor sites both *in vivo* and *in vitro* [3] and have therefore been used as antitumor agents for various estrogen-dependent tumors such as mammary carcinomas [4–6]. The effects of antiestrogens on several other carcinomas such as those of colon, rectum, stomach, kidney and bladder and a melanoma, have also been investigated [7]. The presence of specific binding sites for the antiestrogens have been demonstrated in rat liver nuclei [8]. Antiestrogens cause hypertrophy of luminal cells, the inhibition of cell proliferation and an increased rate of cell death in rat uterus [9]. They have also been shown to be potent growth inhibitors of MCF-7 cells [10]. The present report demonstrates the effect of Nafoxidine, 1-(2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl) phenoxy]-ethyl) pyrrolidine on the development of the chemically-induced [11] Zajdela ascitic hepatoma [ZAH]. The effect of Naf on the binding of labelled estradiol to ZAH cells has also been studied.

MATERIALS AND METHODS

Materials

Nafoxidine was a gift from Upjohn Co., U.S.A. 2, 4, 6, 7-[^{3}H] estradiol, 90-100 Ci/mmol (CEA, Saclay) was obtained through the courtesy of Polypeptide Hormones Research Laboratory, Gif-sur-Yvette, France.

Animals and tumor

ZAH cells ($4–6 \times 10^6$) treated with Naf as described below or untreated were injected into the peritoneal cavity of 2.5–3.0 month-old male albino Wistar rats obtained from an inbred colony of this laboratory. The control rats developed the tumor and died within 6–10 days. The ZAH cells were counted on a hemocytometer and their viability was checked by the trypan blue exclusion method.

Treatment with antiestrogen

Naf and estradiol were dissolved in ethanol and diluted to the required concentrations with the ascitic tumor fluid just before injection into the peritoneal cavity. The final concentration of ethanol was always below 1%. For tumor regression studies, Naf solution was mixed with sesame oil and the suspension was injected into the peritoneal cavity of the rats.

Binding of labelled estradiol

ZAH cells washed with PBS were incubated with [^{3}H] estradiol (10 pmol/ml) in CMRL-HEPES-glucose medium at 4° C for 4 hr or 37° C for 15 min in the presence or the absence of the required amount of cold estradiol or Naf. After incubation, the cells were washed thoroughly with PBS and the bound radioactivity counted after solubilizing with 0.2 ml of Soluene-350.

RESULTS

The survival time of rats was prolonged by more than 100 days and 90% of the animals did not

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develop the tumor when they were injected with Naf (1 μM)-treated cells (Table 1); only about 10% of the rats developed the tumor. Estradiol (100 nM) partially reversed the effect of Naf (1 μM); ZAH cells when treated with estradiol (100 nM) alone behaved as in the control group.

The ZAH tumor is visible from the third day of the tumor transplantation; if Naf was injected at this stage into the peritoneal cavity, there was regression of the tumor (Table 2). Tumor regression was observed in about 70–80% of the cases, when 100–400 μg of Naf was injected per rat.

The binding of [^{3}H] estradiol to ZAH cells, both at 4° C and 37° C, was competitively inhibited in the presence of different concentrations of cold estradiol (Fig. 1A). Naf also inhibited the binding of labelled estradiol to ZAH cells at 37° C (Fig. 1B).

Figure 2 shows the viability of ZAH cells when incubated with Naf for different time intervals. After 48 hr incubation, the viability of cells decreased to about 10% with Naf (10 μM).

DISCUSSION

The results presented above demonstrate the antitumor activity of Naf against an ascitic form of rat hepatoma. Nafoxidine and tamoxifen have been shown to have antitumor activity against various types of breast cancers [12]. Antiestrogen therapy has been used in cancers where the tumor is estrogen responsive. Although liver is known to have estrogen receptors [1] and our results (Fig. 1) demonstrate the presence of estrogen binding sites on the ZAH cells, no attempt has been made earlier to use antiestrogens in the case of hepatomas. However, progestin has been used in the therapy for human hepatomas and the regression of tumor in one out of three cases has been observed [2]. The requirement of estrogens for the growth of normal cells is well known. Liver cells also appear to require estrogens in the culture medium for their optimal growth *in vitro* [13]. In addition to the longer survival time of rats in the case of cells treated with Naf (Table 1), there was no development of tumor in about 90% animals. Nafoxidine also induced the regression of an already developed tumor (Table 2). Naf, however, does not have 100% antitumor activity against ZAH (Tables 1 and 2). About 10% of the Naf-treated rats still developed the tumor; therefore, the role of some host factors modulating Naf action or development of the tumor cannot be ruled out. However, Naf (10 μM) possessed high cytotoxic activity for ZAH cells (Fig. 2). Similar results have been shown when growth of MCF-7 cells was inhibited by Naf at higher doses [14].

Antiestrogens either bind to the estrogen receptors thereby competing with estrogen for the binding [15], or have specific binding sites [16].

*Table 1. Development of Zajdela ascitic hepatoma in rats after treatment with nafoxidine**

	Number of rats	Survival time after the injection of antiestrogen (days)			
		< 10	11–20	21–60	> 100
Control†	30	30	–	–	–
Nafoxidine (1 μM)	30	–	3	–	27
Nafoxidine (1 μM) + Estradiol-17β (100 nM)	30	–	9	21	–
Estradiol-17β (100 nM)	10	10	–	–	–

*Ascitic tumor was mixed with the required concentrations of nafoxidine or estradiol just before injecting it into the peritoneal cavity.
†Control rats were injected with the ascitic tumor treated with the same concentration of ethanol as was used for the antiestrogen-treated animals.

*Table 2. Regression of Zajdela ascitic hepatoma by Nafoxidine**

Group	Animals per group	Animals with development of tumor	Animals in which tumor regressed†
Control	20	20	0
Nafoxidine (100 μg)	20	6	14
Nafoxidine (400 μg)	20	2	18

*Nafoxidine was injected once into the peritoneal cavity of the rats on the 3rd day after transplantation of the tumor, when a slight bulge was observed due to tumor progression.
†The tumor was taken to be regressed when the rats survived more than 60 days and none of the usual symptoms due to the presence of tumor were observed.

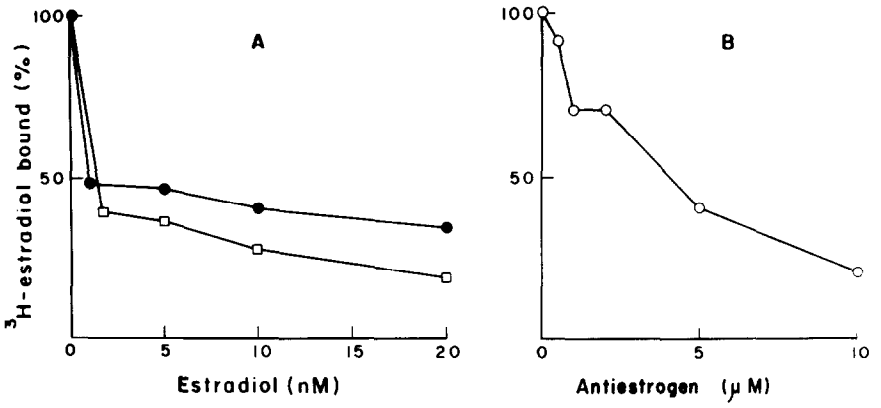

Fig. 1. (A) Effect of cold estradiol on the binding of [^{3}H] estradiol to 10^6 ZAH cells at 4° C for 4hr (□) and 37° C for 15 min (●). (B) Effect of Naf (○) on the binding of labelled estradiol to ZAH cells at 37° C.

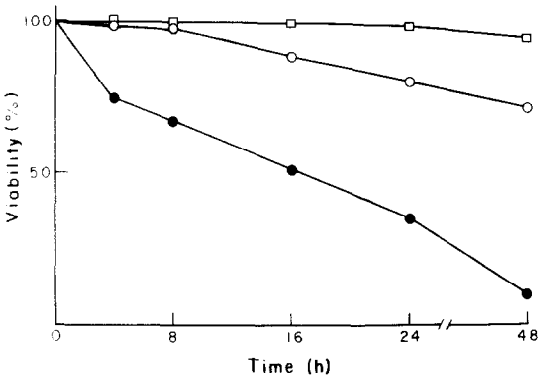

Fig. 2. Effect of Naf on the viability of ZAH cells up to 48 hr incubation in DME-glucose medium; □, control; ○ Naf (1 μM); ●, Naf (10 μM).

Previous studies on fetal uterus have suggested the binding of Naf and tamoxifen to the same site where estradiol binds. The relative binding affinity of Naf and tamoxifen to the estrogen receptor was lower than that of estradiol [17]. Antiestrogens have thus been shown to have cytotoxic effects *in vivo* and *in vitro* which are not reversed by estrogens [9]. Similar results are reported here where the cell viability is about 10% after a 48hr incubation of ZAH cells with Naf (Fig. 2).

The mechanism by which Naf exerts the anti-tumor activity on ZAH may be a two way process, involving competition for the binding of an estrogen as well as a cytotoxic effect. Non-steroidal antiestrogens may thereby be the potential anti-tumor agents against various types of hepatomas.

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