ISOLATION OF FATTY ACIDS WITH ANTITUMOR ACTIVITY FROM FUNGAL MYCELIA

STUDIES ON ANTIVIRAL AND ANTITUMOR ANTIBIOTICS. VII

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In our screening for antitumor antibiotics using the EHRLICH ascites tumor, acetone extracts of the mycelia of *Penicillium crustosum*, *Penicillium tardum*, *Cephalosporium diospyri* and *Sepedonium ampullosporum* showed significant antitumor activity. These fungi were selected because of cytotoxicity or antiviral activity on chick embryo fibroblast cell monolayers. The extracts were fractionated by silica gel column chromatography and the active principles were obtained as colorless oils which showed the characteristic infrared absorption bands of long-chain fatty acids. The active principles exert marked antitumor activity against EHRLICH ascites tumor, although they showed neither antiviral activity nor inhibitory activity against microorganisms.

In our antitumor and antiviral antibiotic screening, we obtained colorless oily substances with remarkable antitumor activity against EHRLICH ascites tumor from the mycelia of *Penicillium crustosum*, *Pen. tardum*, *Cephalosporium diospyri* and *Sepedonium ampullosporum*. All of these organisms belonging to the Fungi Imperfecti have been preserved as type cultures in our laboratory. In a primary screen the acetone extracts of the mycelia of these fungi showed either cytotoxicity or antiviral activity on chick embryo fibroblast monolayer (CEF)[1]. The extracts were concentrated *in vacuo* to remove acetone and the resultant suspensions were injected into EHRLICH ascites tumor bearing mice 24 hours after implantation. The antitumor activity was determined from the survival time and body weight gains on the 7th day after implantation. These four organisms were selected because of prolongation of life-span and suppression of the tumor growth. The active principles were fractionated by silica gel chromatography and isolated as oils. The infrared and nuclear magnetic resonance spectra of these were essentially the same, having the characteristics of long-chain fatty acids. In this paper the production and some biological properties of these fatty acids are described.

Production and Isolation from *Penicillium crustosum* and *Pen. tardum*

The acetone extracts of these two fungi showed antiviral activity *in vitro* in a primary screen. At an early stage in this investigation it was considered that the antiviral active principles might be responsible for the antitumor activity. Thus both

the *in vitro* antiviral activity and *in vivo* antitumor activity were determined of all preparations.

The fermentations and the procedures for extractions and isolations were the same for these two organisms. Fig. 1 shows the procedure for *Pen. tardum*. The fungi have been preserved in the form of slants of a CZAPEK Dox medium. *Pen. tardum* was grown in 500 ml Ehrlemeyer flasks containing 100 ml of the medium composed of (w/v, %) glucose 10, peptone 0.5, $NaNO_3$ 0.1, KH_2PO_4 0.06, $MgSO_4$ 0.04, KCl 0.05 and $CaCO_3$ 1, pH 6.2~6.3 in tap water. The flasks were incubated for 4 days at 27°C on a rotatory shaker. Three hundred ml of the fermented broth were used to inoculate a 100-liter tank containing 60 liters of medium with the same composition as the inoculation medium. The fermentor was stirred at 250 rpm and aerated at a rate of 35 liters per minute for 3 days at 27°C. The fractionation procedure is described in Fig. 1. The mycelium was separated by filtration with Celite and extracted with acetone (20 liters) overnight at room temperature. The extract obtained by filtration was concentrated *in vacuo* to remove the acetone, and ethylacetate was added to the residue to extract the active principle. No antitumor or antiviral activity remained in the aqueous phase after extraction. The ethylacetate layer was dehydrated with anhydrous sodium sulfate and concentrated *in vacuo* to a small volume. The residual oil exhibited both *in vivo* antitumor activity and *in vivo* antiviral activity.

The oil contained several components when it was developed on thin-layer chromatography. Silica gel column chromatography was applied to the oil. A fractionation column of 36 mm in diameter and 100 cm in length was prepared with a mixture of silica gel (Malinckrodt, 100 mesh) 80 g and Celite 545 20 g suspended in hexane. The oil was charged at the top of the column and then the column was developed with hexane (1 liter). The collected eluate was concentrated *in vacuo* to remove the solvent. The colorless oily residue obtained was designated fraction I. Fraction I from *Pen. tardum* was nearly pure fatty acids whereas some triglyceride was present as a minor component in the fraction I from *Pen. crustosum*. This fraction contained almost the total EHRLICH ascites antitumor activity found in the original crude extract, but it showed neither cytotoxicity nor antiviral activity on CEF in the agar diffusion method. Subsequent fractions, II and III were

Fig. 1. Isolation of the antitumor active principle from the mycelium of *Penicillium tardum*

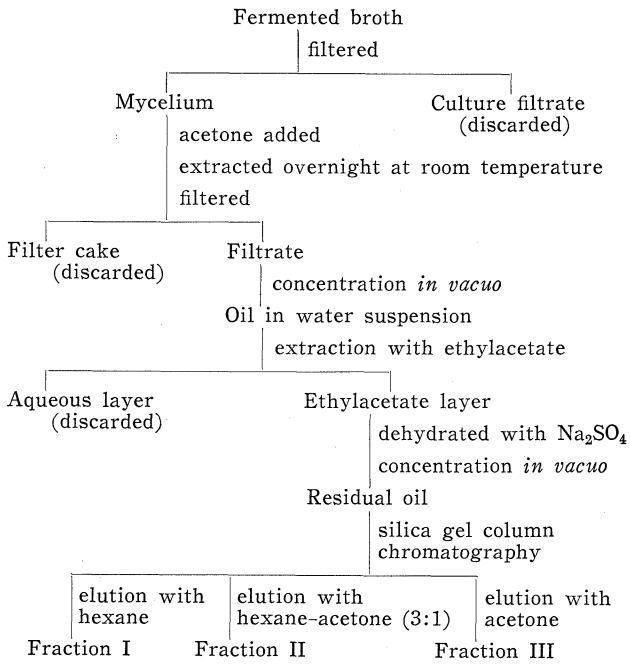

Table 1. Antitumor and antiviral activities of each fraction

Sources	Activities					
	Fraction I		Fraction II		Fraction III	
	Antitumor	Antiviral	Antitumor	Antiviral	Antitumor	Antiviral
Penicillium crustosum	+	−	−	−	−	−
Penicillium tardum	+	−	−	+	−	−
Cephalosporium diospyri	+	−	−	+	−	−
Sepedonium ampullosporum	+	−	+	−	−	+

The crude acetone extracts were fractionated through silica gel column. Fraction I; hexane eluted fraction. Fraction II; hexane - acetone (3 : 1) eluted fraction. Fraction III; acetone eluted fraction. EHRLICH ascites tumor-mouse system was used for determination of antitumor activity. Antiviral activity was assayed as previously reported[2].

eluted with more polar solvent systems, and were found to be inactive against the tumor. It was concluded that the fraction I fatty acids were responsible for the antitumor activity of the original acetone extract. The antitumor and antiviral properties of the fractions are shown in Table 1.

Production and Isolation from *Cephalosporium diospyri* and *Sepedonium ampullosporum*

These organisms were selected first because of activity exhibited in the *in vitro* antiviral test and subsequently in the *in vivo* antitumor test. The fermentations and the procedures for extraction and isolation were essentially the same as those for *Pen. tardum* except the medium which was as previously reported[2]. These two fungi grew in minute pellets in submerged culture. The active principles, obtained from the mycelia as colorless oils, and showed the typical infrared absorption bands of long-chain fatty acids.

Antitumor Activity of Fatty Acid Fractions

Our fatty acids showed antitumor activity against the tumor, as demonstrated in Table 2. It was observed that the maximal tolerated doses of these were 30 mg/mouse/day according to our dose-schedule. The dose is extremely high as compared with other antitumor agents. The minimal inhibitory doses were approximately 3~5 mg/mouse/day so that the chemotherapeutic index is relatively high. The treated mice were sacrificed 30 days after the tumor implantation and examined for solid tumors. It was observed that some of the treated mice were completely cured of both solid and ascites forms of the tumor whereas others had solid tumors even though there was no evidence of the ascites tumor. It is evident that these fatty acid fractions obtained exert significant antitumor activity against EHRLICH ascites carcinoma but the effectiveness against the solid tumor is not established.

Table 2. Antitumor activity of fatty acids produced by some fungi

Fungi	Antitumor activity in screening survival time (days)		Autitumor activity of fatty acids					
			Dose (mg/mouse/day)	Implantation after 7 days			Survival time (days)	
				Tumor		Body wt. gain(g)		
Penicillium crustosum	>30	>30	14	−	−	+4.9	+3.8	>30	>30
	>30	>30	3.5	−	−	+6.5	+4.3	20	27
Penicillium tardum	>30	>30	20	−	−	+2.2	+2.0	>30	>30
	>30	28	5	−	−	+4.0	+4.0	>30	23
Sepedonium ampullosporum	>30	22	20	−	−	+1.5	−0.6	23	>30
	>30	29	5	−	−	+1.6	+1.5	>30	21
Cephalosporium diospyri	>30	>30	16	−	−	−0.2	+1.5	27	27
	>30	21	4	−	−	+3.4	+4.6	>30	21
Control	15.7 (average)			+++		+8.9 (average)		15.7 (average)	

Five-week mice (strain *ddY*) weighing 18~22 g were used in this experiment. Two mice were used under each dose. The fatty acids were administered as homogenized suspensions in distilled water (Tween 80 used for dispersion). The treatment was initiated 24 hours after intraperitoneal implantation of 2×10^6 EHRLICH ascites tumor cells, the fatty acids being given intraperitoneally once daily for 5 consecutive days in a total volume of 0.2 ml. The degree of ascites tumor growth; +++ indicates marked tumor growth, ++ moderate growth, + slight growth, − indicates no growth.

Discussion

The primary sites of action of most antitumor agents are in nucleic acid metabolism. A few antitumor agents are known to have other modes of action, *e. g.*, the glutarimide antibiotics and puromycin. We considered the possibility that the lipids of microorganisms might contain unknown class of antitumor antibiotics. Thus, the acetone extracts of the fungal mycelia were used for screening. The primary screening system used was an agar diffusion method for antiviral activity and the secondary screen involved the EHRLICH ascites carcinoma-mouse system. Our observation indicated that the fatty acids from some fungal mycelia exert significant antitumor activity against the tumor system used. This is the first report that fatty acids showed antitumor activity *in vivo*.

According to a CCNSC report[3], saturated long-chain fatty acids such as stearic (C_{18}), palmitic (C_{16}), myristic (C_{14}) and lauric (C_{12}) acids, were inactive against the solid form of sarcoma-180, adenocarcinoma Ca-755 and ascites form of L-1210. The differences in effectiveness are due to the tumors used and the methods of administrations. The determination of biological activity of lipids depends on the form in which the lipids are added to the assay systems. For example, long-chain fatty acids are known to show hemolytic activity, although under certain condition we found no hemolytic activity. A strong hemolytic agent, lysolecithin, becomes inactive when it is solubilized by deoxycholate. In these studies we observed that the active principles were less effective against EHRLICH ascites tumor when administered in 20 % propylene glycol solution.

The fatty acid compositions of fungal lipids has been extensively studied by gas chromatography[4]. The major constituent fatty acids are palmitic, oleic and linoleic acids. Members of the *Eurotiales*, in which the ubiquitous *Penicillia* and *Aspergilli* are included, have in general around 20 % linoleic acid, but this can rise to 40 % and even 50 % in some cases. However, free fatty acids extractable from the mycelia with acetone had little been studied so far. Our antitumor active fatty acids fractions also consisted of palmitic, oleic and linoleic acids as will be reported in another paper[5]. Thus, the normal constituent fatty acids are present in the free fatty acid fraction.

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