MITOCROMIN: AN ANTIBIOTIC WITH ANTITUMOR ACTIVITY

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Mitocromin is an antibiotic composed of an equilibrium mixture of two components A and B. The antibiotic is related to daunomycin which also accompanies mitocromin in the culture broth. Mitocromin has antitumor activity as shown in test systems: WALKER 256 carcinosarcoma in rats and Leukemia P-388 in mice.

A number of antimicrobial agents possess the structural unit which is a derivative of 7, 8, 9, 10-tetrahydrotetracene quinone (5, 12) present in glycosidic union with one or more sugar units. The term anthracyclines was proposed for this group[1]. The recognition that one of the members, daunomycin exhibited some antitumor activity focused fresh attention on this group of compounds[2]. We wish to report the isolation of a new number of this class named mitocromin. In addition to being antibacterial, mitocromin shows significant activity against WALKER 256 carcinosarcoma in rats and leukemia P-388 in mice.

Mitocromin is produced by two unidentified species of *Streptomyces*, designated as B 35251 and B 105621. The cultures are maintained in the usual complex natural media, a typical example being the following: glucose 1 %, soy bean meal 1.5 %, distillers' solubles 0.5 %, dibasic potassium phosphate 0.25 %, sodium chloride 0.1 % and calcium carbonate 0.1 %. The progress of the fermentation was followed by means of a disc plate assay using *B. subtilis*.

Preliminary experiments indicated that the active principle was basic, associated with the red pigment and partially extractable into organic solvents such as *n*-butanol or chloroform. The active material was also shown to be a mixture of several components which respond differently to different assay procedures. The recovery was evaluated on the basis of both microbiological and spectrophotometric assays. At various stages, an *in vivo* antitumor assay was also employed in order to observe possible correlation among the three methods.

For recovery, the culture was stirred at pH 4 before filtration to minimize the occlusion of activity by the mycelium. The filtered broth was extracted once with a mixture of chloroform and isopropanol (9 : 1) in the presence of 10 % sodium chloride. The extract, which contained the bulk of the antitumor activity, was concentrated to a thick syrup and then shaken between isopropyl ether and 10 % aqueous methanol with 1 % acetic acid. After three extractions with fresh aqueous methanolic acetic

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acid, the combined aqueous layer was concentrated partially and re-extracted with 9 : 1 chloroform-isopropanol in the presence of 10 % sodium chloride. The solvent extract was concentrated to near dryness and the residue triturated with isopropyl ether. The dark red solid thus obtained was active in the WALKER-256 tumor system in the dose range of 0.1~0.2 mg/kg.

The crude antibiotic showed about 10~15 orange red to purple spots in thin layer chromatography. By paper chromatography followed by bioassay, four active components could be seen: A, B, C and D with Rf values of 0.6, 0.5, 0.3 and 0.05. The first two are the major components followed by D and C in decreasing order of abundance. When the fermentations were carried out beyond the peak of activity, the D component was seen to predominate.

For purification, several methods based on countercurrent distribution or chromatography appeared suitable. However, the active principle showed considerable sensitivity in solution as indicated by the formation of additional entities representing

Fig. 1. Infrared spectrum of mitocromin

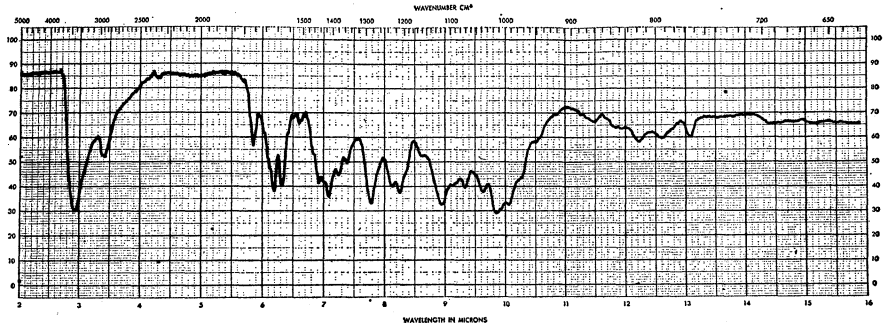

Table 1. Properties of mitocromin

Appearance	Bright red amorphous powder, m. p. 170~175°C
Analysis	Found: C 55.66, H 6.97, N 2.32
U. V. Spectrum (methanol)	234 mμ $E^{1\%}_{1cm}$ 465 252 mμ $E^{1\%}_{1cm}$ 317 290 mμ $E^{1\%}_{1cm}$ 114
IR Spectrum (KBr)	3390, 2941, 1709, 1613, 1410, 1379, 1351, 1284, 1212, 1117, 1095 and 1014 cm^{-1}
Chromatographic behavior:	
A. Thin-layer	Chloroform-methanol (9 : 1) with 1% formic acid, silica gel plate, Rf 0.2~0.3
B. Paper	Solvent phase of benzene-chloroform acetic acid-water (2 : 2 : 1 : 1) and wet paper, Rf: A-0.5, B-0.3

Table 2. Antitumor activity of mitocromin*

	Dose mg/kg	No. of Survivors	T/C %
WALKER 256	0.01	5/6	8
	0.008	5/6	28
	0.006	5/6	42
	0.004	6/6	32
	0.008	6/6	15
	0.004	6/6	32
			% Increase in survival time
Leukemia P-388	0.008		172
	0.004		150
	0.002		145
	0.001		118
	0.008		175
	0.006		185
	0.004		185
	0.002		175

* These results were obtained through the courtesy of Dr. T. J. MCBRIDE, The John L. Smith Memorial for Cancer Research, Maywood, New Jersey.

either degradation or transformation products. Purification by countercurrent distribution was studied using the following two systems: *n*-butanol-ethyl acetate-1% aqueous acetic acid (1:3:4) and ethanol-chloroform-0.2% aqueous acetic acid (15:84:100). In both systems the D component favored the aqueous phase, while A and B were intermediate and the neutral components the solvent phase. The D component could be separated readily from the others and subsequently obtained as a crystalline solid. Its properties as well as comparison with an authentic sample showed that it was identical with the known antibiotic daunomycin. Since the crude mitocromin was much more active than crystalline daunomycin and since the C component was only of minor significance, it appeared that either or both of the components A and B must be responsible for the activity.

Table 3. Antibacterial activity of mitocromin*

Organism	M.I.C. (mcg/ml)
Staphylococcus aureus	0.045
Staphylococcus faecalis	0.19
Escherichia coli	3.12
Salmonella typhosa	3.12
Klebsiella pneumoniae	1.56

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For the purification of the components A and B, methods based on partition chromatography appeared to offer several advantages over those based on countercurrent distribution described above. Two such procedures, somewhat related to each other, were found to be suitable for this purpose. In one, a mixture of ethyl acetate with 8~12% of *n*-butanol served as the mobile phase, with 1% acetic acid as stationary phase and carboxymethyl cellulose as the support. In this procedure, the neutral and weakly basic fractions appeared first, followed by components A, B, C and D. The components A and B could be separated from the other members but not from each other. In an attempt to separate these two, the second column procedure was studied in which a mixture of benzene, chloroform and ethanol (5:5:1) formed the mobile phase with 0.2% acetic acid as stationary phase and carboxymethyl cellulose as the support. The components A and B were eluted in reverse order from this column but they were still very close to each other. After several attempts it became evident that even though some degree of separation could be achieved by the column, the fractions, when processed, showed only mixtures comparable to the starting sample. The possibility that the two componets A and B might be existing in a mobile equilibrium was therefore considered. In order to verify this, the sample was first separated into the two components by paper chromatography. The fractions were recovered and resubjected to paper chromatography. Appearance of both components in the purified samples indicated the presence of the equilibrium A $\rightleftharpoons$ B. In view of this problem, it was decided to obtain a purified sample of a mixture of the two for preliminary characterization. A more precise characterization will become possible with the understanding of some of their structural features. The name mitocromin was applied to the A—B mixture and some of the physical properties of a purified mixture are given in Table 1. The infrared spectrum of mitocromin run as KBr pellet is shown in Fig. 1.

Both components A and B of mitocromin appear to be equally active against *B.*

subtilis and based on a number of *in vivo* tests carried out on diverse samples (of mixtures), they may have comparable antitumor activity also. Although a complete description of the antitumor activity will not be given here, typical examples are shown to illustrate its nature and extent in Table 2.

Mitocromin is highly cytotoxic to HeLa cells grown in cell culture in the concentration range of 0.001~0.005 mcg/ml. Table 3 illustrates the antibacterial activity of mitocromin.

References

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