Vol. 94, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 16, 1980 Pages 769-776

PURIFICATION AND CHARACTERIZATION OF MACROMOMYCIN-I, A PROTEIN ANTIBIOTIC CONTAINING A NON-PROTEIN CHROMOPHORE

Jan M. Woynarowski*† and Terry A. Beerman*

*Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14263, U.S.A.
†Permanent Address: Department of Pharmaceutical Technology and Biochemistry Technical University of Gdansk, 80-952 Gdansk, Poland

Received April 21,1980

Summary

One-step chromatography of crude macromomycin on DEAE-Sephacel yielded two forms of the antibiotic. The more active form of the drug contained a non-protein chromophore. Apoprotein and chromophore components of this drug form were separated. The apoprotein was found to be inactive and the chromophore only slightly active against Sarcina lutea, while recombination of both components resulted in a partial recovery of the activity. In contrast, recombination of chromophore and apoprotein did not restore the ability of intact macromomycin to nick isolated DNA.

Macromomycin, an antitumor antibiotic, is an acidic protein with a molecular weight of around 12,000 daltons (1). The drug was shown to inhibit growth of several experimental tumors in vivo (2), cultured tumor cells (3) and Gram-positive bacteria (4). Macromomycin was found to induce breaks in cellular DNA (5) as well as in cell free DNA (6-8). The drug's ability to damage cellular DNA seems to be related to inhibition of DNA synthesis and inhibition of cell growth (5).

Macromomycin has been purified to various degrees by several different procedures (1, 6, 7). Recently, it was reported that the drug exists in two forms of identical molecular weight, isoelectric point and amino acid composition, but that one (referred to as auromomycin) contains a 350 nm absorbing chromophore (8).

For our studies on the mode of action of macromomycin, we needed a simple method to obtain purified and better defined drug. In this paper, we describe a new, one-step procedure which allows isolation of the antibiotic from crude preparation of macromomycin and also enables separation of the chromophore containing form of the drug from the one that lacks chromophore.

0006-291X/80/110769-08$01.00/0

The former, most likely corresponding to auromomycin, is designated as macromomycin-I (MCR-I)[1], while the latter is referred to as macromomycin-II (MCR-II). In addition, we separated the chromophore from the protein part of MCR-I to explore the contribution of both components to the biological activity of the antibiotic.

MATERIALS AND METHODS

Crude macromomycin (NSC #170105) was provided by Developmental Program Chemotherapy, of the National Cancer Institute. All steps of the isolation procedure were done at 5°C in partial or complete darkness. Crude material (1.5 g) was applied on a column (3 x 80 cm) packed with DEAE-Sephacel (Pharmacia, Sweden), and equilibrated with 10 mM Tris-HCl, pH 7.9. The column was eluted (80 ml/hr) initially with the equilibrating buffer followed by elution with a two-step linear gradient of NaCl concentration (0-10 mM NaCl in 250 ml and 10-20 mM NaCl in 2000 ml, respectively). Elution of protein was monitored by absorbtion at 280 nm (Gilson UV monitor), and the salt gradient was determined by measuring conductivity of the collected fractions. Antibacterial activity against Sarcina lutea was measured by a disc method (4). Drug damage to isolated DNA was assayed by measuring conversion of Form I PM2 DNA into Form II based on the loss of fluorescense of nicked PM2 DNA-ethidium bromide complex in alkali (9). PM2 DNA was incubated with drug for 30 min at 37°, and results were expressed as decrease of fluorescense in treated samples normalized with respect to the fluorescense of control samples (10). Cytotoxic activity of MCR-I and MCR-II against HeLa and L1210 cells was determined as described previously (5). The active fractions were pooled as indicated in Fig. 1, concentrated by ultrafiltration on Amicon P-10 membrane and stored at -20°C. The amount of protein in MCR-I and MCR-II preparations was found to be 8 mg and 17 mg, respectively, as estimated by Lowry method (11) using bovine serum albumine for calibration curve. Separation of MCR-I into a 350 nm chromophore and protein components was done by extraction of freeze-dried material with methanol as described for neocarzinostatin (12). UV spectra were recorded on a Cary 118 spectrophotometer.

RESULTS AND DISCUSSION

Preliminary fractionation of crude macromomycin on DEAE-Sephacel showed that the drug's biological activities (inhibition of HeLa and Sarcina lutea cell growth and strand scission of cellular as well as isolated DNA) were associated with one protein peak, while other components were inactive (Fig. 1A). Chromatography of crude antibiotic under improved conditions (longer column and a two-step salt gradient with a shallow slope for the second step) revealed that the active material consisted of at

[1]Abbreviations: MCR-I, macromomycin-I; MCR-II, macromomycin-II; Tris, tris(hydroxymethyl) aminomethane; DTT, dithiothreitol; Form I PM2 DNA, covalently closed duplex DNA; Form II PM2 DNA, nicked circular duplex DNA.

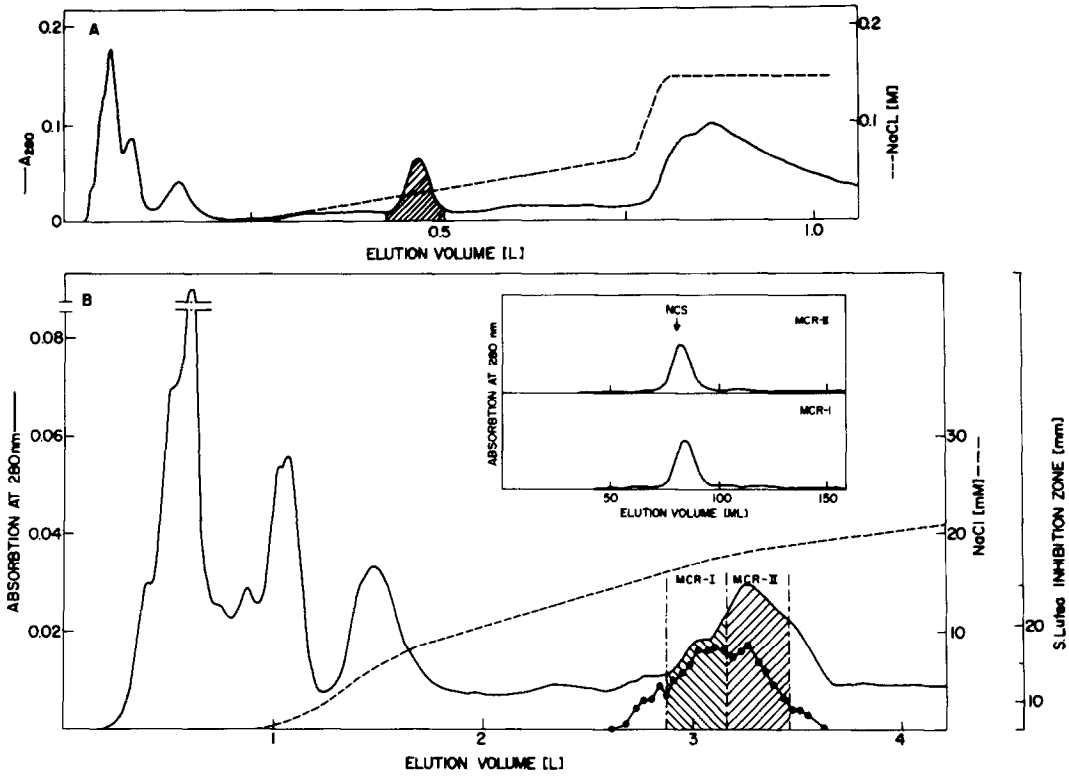

Fig. 1. Chromatography of crude MCR on DEAE-Sephacel columns. Panel A: 300 mg sample was fractionated on a 1.5 x 25 cm column eluted at 52 ml/h with 10 mM Tris, pH 7.9 and a gradient of NaCl as shown in the figure. Shaded area indicates fractions which inhibited the growth of HeLa and Sarcina lutea cells and nicked cellular DNA in HeLa cells. Panel B: 1.5 g sample was fractionated on a 3 x 80 cm column as described in Materials and Methods. Solid circles show activity of the collected fractions (at 2 µl/disc) against Sarcina lutea. Fractions were pooled as indicated by shaded areas and designated MCR-I (shoulder) and MCR-II (main peak). Insert shows elution profiles of MCR-I and MCR-II on a Sephadex G-50 column (1.5 x 75 cm) eluted with 10 mM Tris, pH 7.9 at 20 ml/hr. An arrow indicates the elution volume of neocarzinostatin (NCS).

least two substances (Fig. 1B). Under these conditions macromomycin was eluted as a broad peak with a distinct shoulder at 17-19 mM NaCl (Fig. 1B). Fractions corresponding to the shoulder and the main peak were pooled as indicated in Fig. 1B and are further referred to as MCR-I and MCR-II, respectively. Both MCR-I and MCR-II banded at the same position upon electrophoresis in 15% acrylamide gel (not shown) and when analyzed by chromatography on Sephadex G-50 (Fig. 1B). The elution volumes of MCR-I and MCR-II were both close to the elution volume of neocarzinostatin [MW 10,700 (13)]. A similar value of molecular weight of around 12,000 was estimated pre-

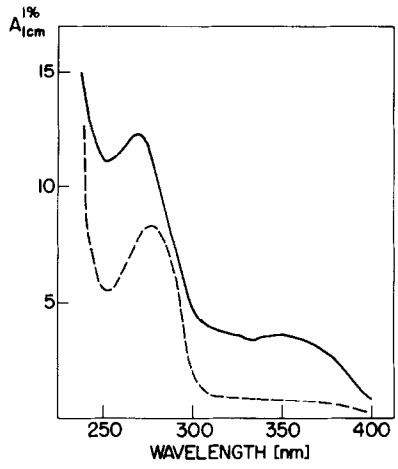

Fig. 2. UV spectra of MCR-I (——) and MCR-II (---) in 1 mM Tris, pH 7.9.

viously for macromomycin (1) and auromomycin (8). Isoelectric focusing indicated the isoelectric point of MCR-I and MCR-II to be 5.4 as was reported for chromophore-lacking macromomycin and auromomycin (1, 8). UV spectra of MCR-I and MCR-II differ significantly (Fig. 2). MCR-II showed a typical protein spectrum with a maximum at 280 nm and almost no absorbtion above 300 nm. In contrast, the maximum of the UV spectrum of MCR-I is shifted to 267 nm and there is an additional broad band centered at 350 nm indicating the presence of non-protein chromophore. The entire spectrum of MCR-I is very similar to that reported for auromomycin (8) while the spectrum of MCR-II resembles the spectrum of previously isolated chromophore-lacking macromomycin (1, 8). Substances referred to previously as macromomycin and auromomycin have been isolated from the same microorganism but by separate procedures. Our results indicate that the crude preparation of macromomycin contains a substance probably identical to auromomycin as well as the chromophore-lacking antibiotic. It seems very likely that much of the published data on "macromomycin" was obtained with the preparations containing a mixture of the drug with and without chromophore. Auromomycin (and thus presumably MCR-I) contains the same protein as chromophore-lacking macromomycin (8). In this light both substances should be considered as two forms of the same drug rather than two different antibiotics.

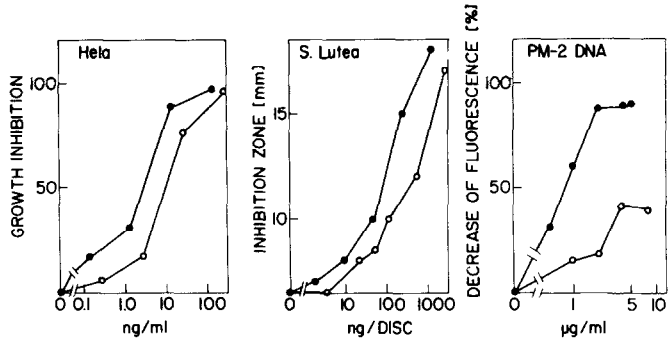

Fig. 3. Biological and biochemical activities of MCR-I (-●-) and MCR-II (-o-). Left panel shows growth inhibition of HeLa cells; middle panel, growth inhibition of Sarcina lutea, and right panel, ability to nick isolated PM2 DNA.

Using the extinction coeffecients of auromomycin and chromophore-lacking macromomycin (8), we estimated that MCR-I consists of about 80% of the chromophore-containing drug, while MCR-II contains about 85% of the chromophore-lacking form. Though the biological activity of MCR-II could be enhanced by the presence of MCR-I, the activity elution profile (Fig. 1) indicates that MCR-II has antibiotic properites by itself. MCR-I inhibited growth of Sarcina lutea at 2-10 ng/disc, while 20-50 ng/disc was needed in the case of MCR-II to produce a similar effect (Fig. 3). Cytotoxic activity of MCR-I and MCR-II against HeLa cells (Fig. 3) was 2.9 and 9.4 ng/ml, respectively (ID_{50} values). Similar activities were found against cultured L1210 leukemia cells (data not shown). MCR-I was able to completely convert Form I PM2 DNA to Form II with 50% of the effect at 0.7 µg/ml. MCR-II also cut PM2 DNA, but the maximal effect observed was only 40% conversion at drug levels of 4-8 µg/ml (Fig. 3). These data are, in general, consistent with those published previously for auromomycin and macromomycin (6-8) and confirm the higher activity of the chromophore-containing form of the drug.

The biological and biochemial activities of macromomycin preparations were reported to be strongly light and temperature sensitive (1, 7). We found that MCR-I loses 15-20% of its antibacterial activity after 3 hr exposure to normal laboratory light, even when the sample was kept in ice.

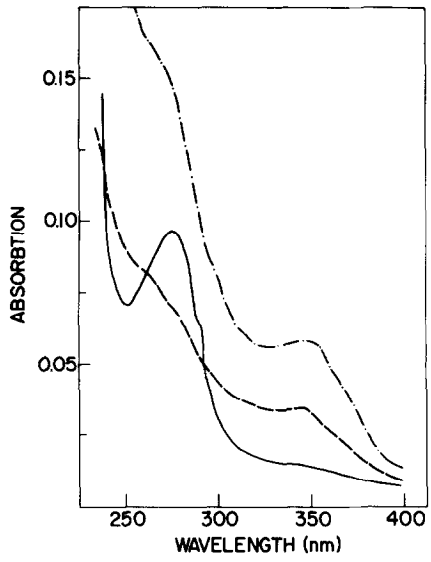

Fig. 4. UV spectra of MCR-I chromophore in methanol (-·-), in 1 mM Tris, pH 7.9 (---) and spectrum of MCR-I apoprotein in 1 mM Tris, pH 7.9 (——). Concentrations were adjusted to correspond to 200 µg/ml of MCR-I.

It has been reported recently that another protein antibiotic, neocarzinostatin, contains a non-protein chromophore, which could be separated from apoprotein by methanol extraction of freeze-dried material (12). We employed the same protocol for MCR-I. Fig. 4 shows the UV spectra of methanol-soluble and methanol-insoluble fractions of MCR-I. The spectrum of the latter resembles that of MCR-II, with a maximum of 275 nm and considerably lower absorption above 350 nm than in the case of intact MCR-I. In contrast, the spectrum of the methanol-soluble material has a maximum at 345 nm, a shoulder at 265-270 nm and increasing absorption at shorter wavelengths (Fig. 4). A very similar spectrum was reported for neocarzinostatin chromophore (12). In addition, MCR-I chromophore absorbs more strongly in methanol than in water (Fig. 4). The molecular weight of MCR-I chromophore seems to be 667 daltons, as suggested by preliminary mass spectroscopy data[2].

In order to explore the role of chromophore and apoprotein components in the biological activity of MCR-I, we studied their ability to inhibit

[2]Mass spectroscopy experiments were performed by Dr. Pawel Kolodziejczyk.

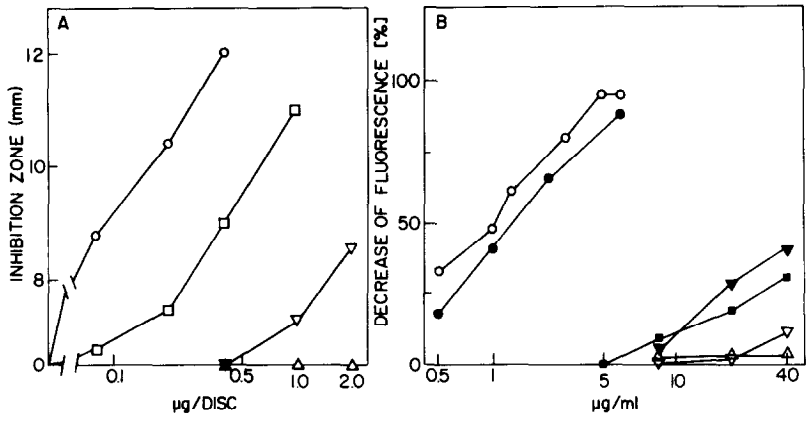

Fig. 5. Effects of MCR-I components on growth of Sarcina lutea (panel A) and the nicking of isolated PM2 DNA (panel B) in absence (open symbols) and presence (solid symbols) of 1 mM DTT. Concentrations of MCR-I components are expressed with respect to the original concentration of MCR-I. Intact MCR-I (-o-, -●-); MCR-I chromophore (-▽-, -▼-); MCR-I apoprotein (-△-); mixture (1:1) of MCR-I chromophore and apoprotein (-□-). Apoprotein with DTT and the mixture of chormophore without DTT showed no activity in the PM2 DNA assay and were omitted from panel B.

the growth of Sarcina lutea (Fig. 5A) and to nick PM2 DNA (Fig. 5B). It appeared that apoprotein has no antibacterial activity even at levels as high as 2 µg/disc. Chromophore showed some activity in this assay, though it was one order of magnitude lower than that of intact MCR-I. However, recombination of chromophore with apoprotein resulted in 20-25% recovery of the original activity (Fig. 5A). Similar stimulation of antibacterial activity (up to 50% of the MCR-I activity) was observed for a mixture (1:1) of MCR-I chromophore with MCR-II. In contrast, separated components of MCR-I were practically inactive when assayed for cutting of PM2 DNA (Fig. 5B). Only slight recovery of activity was observed for chromophore and chromophore-apoprotein mixture when incubation with DNA was carried out in presence of 1 mM DTT (Fig. 5B).

Though the methanol extraction may cause some irreversible changes of drug properties, the results suggest that both chromophore and apoprotein contribute to the in vivo activity of MCR-I. On the other hand, the chromophore seems to have some activity by itself. Further detailed investigations, including determination of chromophore structure, are necessary to

unequivocally establish the role of chromophore and apoprotein in biological activity of MCR-I. Preliminary characteristics hitherto presented may be helpful in these studies. Moreover, the purification procedure described in this paper enables isolation of both chromophore-containing and chromophore-lacking forms of macromomycin and consists of only one step, as opposed to published protocols that involve several chromatographic steps (1, 6-8).

ACKNOWLEDGEMENTS

The authors wish to thank Jeff LaDuca, Janet Arnone, and Pat Dix for excellent technical assistance, and also Nina Ruth Wright for her aid in editing this manuscript. This work was supported in part by National Institute of Health Grants CA-24906 and CA-13038.

REFERENCES

1. Yamashita, T., Naoi, N., Watanabe, K., Takeuchi, T., and Umezawa, H. (1976) J. Antibiot., 29: 415-423.
2. Lippman, M.M., Laster, W.R., Abott, B.J., Venditti, J., and Baratta, M. (1975) Cancer Research, 35: 939-945.
3. Kuminoto, T., Hori, M., and Umezawa, H. (1972) Cancer Research, 32: 1251-1256.
4. Chimura, H., Ishizuka, M., Hamada, M., Hori, S., Humura, K., Iwanage, J., Takeuchi, T., and Umezawa, H. (1968) J. Antibiot., 21: 44-49.
5. Beerman, T.A. (1978) Biochem. Biophys. Res. Commun., 83: 908-914.
6. Sawyer, T.H., Prestayko, A.W., and Crooke, S.T. (1979) Cancer Research 39: 1180-1184.
7. Im, W.B., Chiang, C.K., and Montogomery, R. (1978) J. Biol. Chem., 253: 3259-3264.
8. Yamashita, T., Naoi, M., Hidaka, T., and Watanabe, K. (1979) J. Antibiot., 32: 330-339.
9. Morgan, A.R., and Pulleybank, D.E. (1974) Biochem. Biophys. Res. Commun., 61: 396-403.
10. Beerman, T.A. (1979) Biochim. Biophys. Acta., 564: 361-371.
11. Lowry, O.M., Rosenbrough, N.Y., Farr, A.L., and Randell, R.J. (1951) J. Biol. Chem., 193: 265-275.
12. Napier, M.A., Holmquist, B., Strydom, O.J., and Goldberg, J.H. (1979) Biochem. Biophys. Res. Commun., 89: 635-642.
13. Meinhofer, J., Maeda, H., Glaser, C.B., Czombos, J., and Kuromizu, K. (1972) Science, 178: 875-876.