BIOLOGICAL ACTIVITY OF MACROMOMYCIN

TAKAYOSHI HIDAKA, YOSHIAKI YANO, TOSHIAKI YAMASHITA
and KIYOSHI WATANABE

Biochemical Research Laboratory, Kanegafuchi Chemical Industry Co., Ltd.,
Takasago, Hyogo, Japan

(Received for publication October 9, 1978)

Macromomycin (MCR) is a polypeptide antitumor antibiotic isolated from the culture broth of *Streptomyces macromomyceticus*. Antitumor activities of MCR were examined against three different tumor systems, *i.e.*, EHRLICH ascites carcinoma, L1210 leukemia and LEWIS lung carcinoma. Daily intraperitoneal treatment with MCR for 5 days showed a strong inhibition against EHRLICH ascites carcinoma. Both single and repeated intraperitoneal injections of MCR were effective over a wide dose range against intraperitoneally inoculated L1210 leukemia and MCR intravenously administered was also active against intravenously inoculated L1210 leukemia. Daily local subcutaneous injections of MCR produced the prolongation of life span of mice to which LEWIS lung carcinoma was subcutaneously inoculated with some cured mice, but daily intraperitoneal injections of MCR showed no activity. Single intravenous administration of MCR inhibited early LEWIS lung carcinoma, but not advanced LEWIS lung carcinoma.

The combination of MCR with aracytidine, or cyclophosphamide showed a synergistic activity against L1210 leukemia. MCR was not inactivated by treatment with serum, although neocarzinostatin was markedly inactivated by the same treatment.

The antitumor antibiotic, macromomycin (MCR), first isolated from the culture broth of *Streptomyces macromomyceticus* by CHIMURA *et al.* (1968)[1], was demonstrated to exhibit antitumor activity against L1210 leukemia and Sarcoma ascites 180, and to inhibit the growth of Gram-positive bacteria. The antibiotic is an acidic polypeptide having a molecular weight of about 12,000 and an isoelectric point of pH 5.4[2]. It has been reported by LIPPMAN *et al.*[3] that MCR is active against various mouse tumors such as L1210 leukemia, P388 leukemia, B16 melanoma and LEWIS lung carcinoma.

The mode of action of MCR is unique, since it is thought to bind to the surface of tumor cells and consequently to inhibit DNA synthesis[4,5]. Moreover, the cytotoxicity of MCR to cells can be abolished by brief treatment of cells with trypsin[5,6,7]. SUZUKI *et al.*[8] have reported that MCR causes strand scission of DNA in HeLa and L5178Y cells and inhibits DNA synthesis.

During the course of production and purification of MCR in our laboratory, *S. macromomyceticus* was found to produce a second antitumor antibiotic designated auromomycin whose properties have been characterized[9]. MCR differs from auromomycin in some biological and physicochemical properties and consequently possesses some advantages over auromomycin. Thus, MCR exhibits a lower acute toxicity and displays stronger activity with respect to prolongation of life of mice inoculated with L1210 leukemia.

In this study, we wish to report the antitumor activity of MCR, the effect of combination treatment of MCR with other antitumor agents, and the influence of serum on the antibacterial activity of MCR in comparison with neocarzinostatin.

Materials and Methods

Animals and Tumors

Female mice: BDF_1 (C57BL × DBA/2) weighing 18~22 g, ddY (20~24 g) and C57BL (18~22 g) were used. All mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals and the pellet diet and water were freely given.

EHRLICH ascites carcinoma was supplied by the Institute of Microbial Chemistry, Tokyo, and was maintained in ddY mice by weekly intraperitoneal transplantation. L1210 leukemia was supplied by the Pharmacology Division, National Cancer Center Research Institute, Tokyo, and was maintained in BDF_1 mice by weekly intraperitoneal transplantation. LEWIS lung carcinoma was supplied by the National Cancer Institute, U.S.A., and was maintained in C57BL mice by biweekly subcutaneous transplantation. The solid tumor of 14-day old LEWIS lung carcinoma was excised and minced well with scissors. The minced tumor, suspended in physiological saline, was homogenized gently with a glass homogenizer. The homogenate was filtered first through two layers of sterilized gauze and then through a sterilized stainless steel cytosieve (200 mesh) to remove clumped cells. Viable cells were determined microscopically by the trypan blue exclusion test with the use of a haemocytometer.

Agents

MCR was purified from the culture broth of *S. macromomyceticus* by a slight modification of the method reported previously[2]. A lyophilized powdered form of MCR containing maltose (ratio 1: 19, by weight) was prepared for the purpose of stabilization of the MCR.

Neocarzinostatin (NCS) and cyclocytidine (Cyclo-C) were obtained from Yamanouchi Pharmaceutical Co., mitomycin C (MMC), adriamycin (ADM) and 5-fluorouracil (5-FU) from Kyowa Hakko Kogyo Co., aracytidine (Ara-C) from Nippon Shinyaku Co., carbazilquinone (CQ) from Sankyo Co. and cyclophosphamide (EX) from Shionogi & Co. These agents were dissolved in physiological saline prior to use.

Antitumor Activity of MCR

Tumor cells were inoculated into the mice in the following manner: EHRLICH ascites carcinoma into ddY mice intraperitoneally (2×10^6), L1210 leukemia into BDF_1 mice intraperitoneally (1×10^5) or intravenously (1×10^3 or 1×10^5), LEWIS lung carcinoma into the inguinal region subcutaneously (2×10^6) or into the tail vein of BDF_1 mice intravenously (1×10^5).

MCR was injected into the mice *via* different routes in a volume of 0.1 ml/10 g of body weight. The administration schedule is indicated in each experiment.

Combination of MCR with various antitumor agents

BDF_1 mice were inoculated intraperitoneally with 1×10^5 cells of L1210 leukemia. Each agent was administered alone or in combinaton with MCR intraperitoneally once daily for 5 days, starting 24 hours after tumor inoculation.

Evaluation of antitumor activity

Antitumor activity was evaluated as the increase in life span (ILS% = T/C × 100 − 100) calculated on the basis of the mean survival time (MST) of the treated mice over the control. When two agents were used together, the effect was considered to be synergistic if the combined treatment showed longer ILS (%) than the sum of individual ILS (%) of the two agents.

Influence of human serum on antibacterial activity

Fresh human serum from healthy subjects was employed. In this experiment, MCR was used without maltose. MCR and NCS were dissolved in 25% human serum at a concentration of 50 μg/ml and incubated for 0, 0.5, 1, 2 and 4 hours at 37°C. The residual activity was determined by the cylinder agar plate method using *Sarcina lutea* PCI 1001 as test organism. MCR and NCS, dissolved in physiological saline, at a concentration of 50 μg/ml were incubated for 4 hours at 37°C as control. No loss of activity was observed under these conditions.

Results and Discussion

Antitumor Activity of MCR

The antitumor activity of MCR was examined against three different tumors (EHRLICH ascites carcinoma, L1210 leukemia, and LEWIS lung carcinoma). As shown in Table 1, MCR markedly inhibited EHRLICH ascites carcinoma, with an effective dose range of 0.2~6.25 mg/kg/day × 5. Moreover, the number of 60-day survivors varied from 3~5 per 6 mice at these concentrations of the drug. By contrast, treatment with 12.5~25 mg/kg/day for 5 days resulted in significant death of the mice.

The antitumor activity of MCR against L1210 leukemia was tested *via* i.p.-i.p. system and i.v.-i.v. system as described in Tables 2 and 3. With respect to the i.p.-i.p. system, MCR showed a marked effect over a wide dose range when administered as a single dose or given for a 5 day period (Table 2). Thus the range of effective dose by the criteria of ILS (%) for a single dose was 0.78~25 mg/kg with a maximal increase in life span of 98.8%. On the other hand, if MCR was provided for 5 days the effective dose range was 0.39~12.5 mg/kg/day with a maximal increase in life span of 77.1%. It may be noted that a single injection of MCR in contrast to multiple injections produced a somewhat longer survival time (98.8% *vs* 77.1%) suggesting that the therapeutic effect of MCR against L1210 is greatly influenced by the first injection. As observed in the study with EHRLICH ascites

Table 1. Effect of MCR on survival time of EHRLICH ascites carcinoma (i.p.-i.p. system).

Dose (mg/kg/day)	MST (days)	ILS (%)	60-Day survivors
25	9.5	−47.8	0/6
12.5	27.5	51.1	1/6
6.25	56.0	207.7	4/6
3.13	58.3	220.3	5/6
1.56	48.7	167.6	4/6
0.78	44.0	141.8	3/6
0.39	45.7	151.1	3/6
0.2	44.7	145.6	3/6
0.1	20.2	11.0	0/6
0	18.2	0	0/6

EHRLICH ascites carcinoma (2×10^6 cells) were inoculated intraperitoneally into ddY mice. MCR was injected intraperitoneally once daily for 5 days, starting 24 hours after tumor inoculation.

Table 2. Effect of MCR on survival time of L1210 leukemia (i.p.–i.p. system).

Dose (mg/kg/day)	Day 1 only			Day 1~5		
	MST (days)	ILS (%)	30-Day survivors	MST (days)	ILS (%)	30-Day survivors
25	15.5	86.7	0/6	7.3	−12.0	0/6
12.5	16.5	98.8	1/6	11.7	41.0	0/6
6.25	12.8	54.2	0/6	14.7	77.1	0/6
3.13	12.8	54.2	0/6	14.0	68.7	0/6
1.56	12.5	50.6	0/6	12.8	54.2	0/6
0.78	10.8	30.1	0/6	11.8	42.2	0/6
0.39	9.5	14.5	0/6	10.8	30.1	0/6
0.2	9.0	8.4	0/6	10.0	20.5	0/6
0.1	8.5	2.4	0/6	9.2	10.8	0/6
0	8.3	0	0/6			

L1210 leukemia (1×10^5 cells) were inoculated intraperitoneally into BDF_1 mice on day 0 and MCR was injected intraperitoneally only on day 1 or daily from day 1~5, starting 24 hours after tumor inoculation.

carcinoma (Table 1), daily treatment with MCR (12.5 ~ 25 mg/kg/day) was toxic to the mice with the loss of body weight. LIPPMAN *et al.*[3] noted that there was a maximal increase in life span of 37% and 22% for L1210 leukemia treated with MCR on day 1 and 5 and day 1 ~ 9, respectively. In our experiment, the maximal increase in life span was greater than 70% which is twice that reported by LIPPMAN *et al.*[3] The reason for this difference is not clear, but it might be due to a difference in the MCR sample used.

In the i.v.-i.v. system, MCR was injected intravenously 1 hour (day 0), or 24 hours (day 1) after intravenous inoculation of 1×10^3 or 1×10^5 cells of L1210. As shown in Table 3, MCR was also effective when given by this route of administration. It was found that MCR was nearly as active at an inoculum size of 10^5 *vs* 10^3 cells. However, the degree of effectiveness differed with the time of administration of the drug.

Table 3. Effect of MCR on survival time of L1210 leukemia (i.v.-i.v. system).

Inoculum size	Dose (mg/kg)	Treatment schedule	MST (days)	ILS (%)	30-Day survivors
10^3 cells	20	Day 0	16.5	73.7	1/6
	10	Day 0	12.3	29.5	0/6
	5	Day 0	10.5	10.5	0/6
	20	Day 1	12.5	31.6	0/6
	10	Day 1	10.5	10.5	0/6
	0	—	9.5	0	0/6
10^5 cells	20	Day 0	10.7	59.7	0/6
	10	Day 0	9.3	38.8	0/6
	5	Day 0	7.3	9.0	0/6
	20	Day 1	9.5	41.8	0/6
	10	Day 1	7.2	7.5	0/6
	0	—	6.7	0	0/6

Cells (1×10^3 or 1×10^5) of L1210 leukemia were inoculated intravenously into BDF_1 mice. MCR was injected intravenously 1 hour (Day 0), or 24 hours (Day 1) after tumor inoculation.

The effect of MCR on the survival time of mice inoculated subcutaneously or intravenously with LEWIS lung carcinoma was also examined. Table 4 reveals that daily intraperitoneal treatment (day 1 ~ 10) had no influence against LEWIS lung carcinoma, whereas local subcutaneous injection of the drug, at the site of tumor inoculation, produced a striking increase in life span. Thus, 6 and 7 of 12 mice treated with 0.78 and 3 mg/kg/day, respectively, survived 90 days after tumor inoculation. Moreover, autopsies of the surviving mice revealed no evidence of the growth of the primary tumor and no metastases into the lung was observed. Based on these results, the mice were considered cured. LIPPMAN *et al.*[3] have also reported that subcutaneously implanted LEWIS lung carcinoma responds to local subcutaneous injection but not to intraperitoneal treatment with MCR. It has been demonstrated

Table 4. Effect of MCR on survival time of LEWIS lung carcinoma (s.c.-i.p., s.c.-s.c. system).

Dose (mg/kg/day)	Route	MST (days)	ILS (%)	90-Day survivors
3.13	i.p.	26.3	−19.3	0/12
0.78	i.p.	33.5	2.8	0/6
3.13	s.c.	74.1	127.3	7/12
0.78	s.c.	61.9	89.9	6/12
0	—	32.6	0	0/12

Cells(2×10^6) of LEWIS lung carcinoma were inoculated subcutaneously into BDF_1 mice. MCR was injected intraperitoneally (i.p.) or subcutaneously (s.c.) at the site of tumor inoculation once daily for 10 days, starting 24 hours after tumor inoculation.

Table 5. Effect of MCR on survival time of LEWIS lung carcinoma (i.v.-i.v. system).

Dose (mg/kg/day)	Treatment schedule	MST (days)	ILS (%)	60-Day survivors
20	Day 1	40.4	69.7	5/10
10	Day 1	40.8	71.4	3/10
5	Day 1	26.8	12.6	0/5
20	Day 14	31.0	30.3	1/5
10	Day 14	22.8	−4.2	0/5
0	—	23.8	0	0/10

Cells (1×10^5) of LEWIS lung carcinoma were inoculated intravenously into BDF_1 mice on day 0.

MCR was injected intravenously on day 1 only, or on day 14 only.

that LEWIS lung carcinoma implanted intravenously in contrast to subcutaneously or intramuscularly inoculated tumor may be more responsive to antitumor agents[10]. Therefore, the therapeutic effect of MCR administered intravenously on early and advanced stages of LEWIS lung carcinoma implanted intravenously was tested. As shown in Table 5, MCR was effective against the early tumor when injected intravenously on day 1, but it had far less activity against the advanced tumor when administered on day 14. All the mice that died by the 60th day had pulmonary metastases.

In these studies, MCR was active against EHRLICH ascites carcinoma, L1210 leukemia and LEWIS lung carcinoma. In addition to these tumors, MCR has been reported to be active against P388 leukemia and B16 melanoma[8]. These results indicate that the agent has a broad antitumor spectrum. Therefore, it would be of interest to test further the antitumor activity of MCR against various other experimental tumors. The antitumor effect of MCR may be due to a direct cytotoxic action on tumor cells, since it has been reported that MCR inhibits the growth of HeLa S3 cells, L5178Y cells[8] and P388 leukemia[11] *in vitro*.

Combination Treatment with MCR and Various Antitumor Agents

The effect of MCR in combination with each of 8 antitumor agents was examined against L1210 leukemia. As shown in Table 6, a synergistic effect of MCR was observed in combination with Ara-C and EX. The combination of MCR with Cyclo-C, NCS, MMC, ADM, 5-FU, or CQ, did not show any significant synergistic effect (data not shown).

Chemotherapy involving various combinations of antitumor agents has been studied in experimental tumor systems and has been used with some success clinically. Therefore, further investigation of the combination of MCR with various other agents will be required to establish the most effective combination.

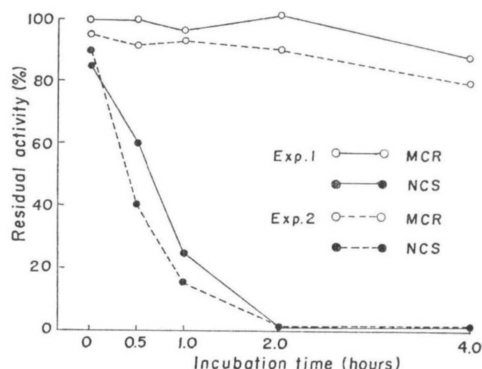
Table 6. Synergistic effect of MCR with Ara-C or EX on survival time of L1210 leukemia.

Agents	Dose (mg/kg/day)	ILS (%)	45-Day survivors
MCR	6	72.3	0/3
	3	62.7	0/6
Ara-C	40	80.7	0/3
	20	72.3	0/3
	10	48.2	0/3
MCR+Ara-C	3+20	165.0	0/3
	3+10	297.6	1/3
EX	50	177.1	0/3
	25	53.0	0/3
	12.5	36.1	0/3
MCR+EX	3+25	310.3	2/3
	3+12.5	48.2	0/3
Saline	—	0	0/6

Cells (1×10^5) of L1210 leukemia were inoculated intraperitoneally into BDF_1 mice. Each agent alone or in combination with MCR was injected intraperitoneally once daily for 5 days starting 24 hours after tumor inoculation.

Fig. 1. Influence of human serum on the antibacterial activity of macromomycin and neocarzinostatin.

Macromomycin (MCR) and neocarzinostatin (NCS) dissolved in 0.9% NaCl were added to 25% human serum to make the final concentration of 50 μg/ml, respectively, and then they were incubated for the indicated time at 37°C. Each sample was placed in the ice until assay. Antibacterial activity was determined by the cylindar agar plate method using *Sarcina lutea* PCI 1001. Experiments were carried out twice.



Influence of Serum on Antibacterial Activity

The results of a study of the influence of human serum on the antibacterial activity of MCR and NCS are shown in Fig. 1. The data reveal that the antibacterial activity of MCR was unaffected by human serum after a 2-hour period at 37°C; moreover, the activity diminished only slightly after 4 hours under the same conditions. In contrast, the antibacterial activity of NCS decreased markedly and by 2 hours no activity was detected. NCS[12,13], as with MCR, is an acidic polypeptide, antitumor antibiotic with a molecular weight of 10,700. It is considered to be chemically similar to MCR, although they differ in amino acid composition. A previous report noted that NCS was inactivated markedly by serum *in vitro*[14]; the mechanism of inactivation or degradation of NCS by serum has also been described[15,16]. The difference in stability of MCR and NCS with respect to human serum may prove to be useful for identification of similar types of antitumor peptides.

Acknowledgements

We wish to express our gratitude to Drs. H. UMEZAWA, T. TAKEUCHI and M. ISHIZUKA of the Institute of Microbial Chemistry for valuable advice and encouragement throughout the course of this study. We also wish to thank Dr. K. KURETANI of the Pharmacology Division, National Cancer Center Research Institute, Tokyo for the supply of L1210 leukemia, and Dr. J. D. DOUROS of the National Cancer Institute, U.S.A. for the supply of LEWIS lung carcinoma.

References

1) CHIMURA, H.; M. ISHIZUKA, M. HAMADA, S. HORI, K. KIMURA, J. IWANAGA, T. TAKEUCHI & H. UMEZAWA: A new antibiotic, macromomycin, exhibiting antitumor activity and antimicrobial activity. J. Antibiotics 21: 44~49, 1968
2) YAMASHITA, T.; N. NAOI, K. WATANABE, T. TAKEUCHI & H. UMEZAWA: Further purification and characterization of macromomycin. J. Antibiotics 29: 415~423, 1976
3) LIPPMAN, M. M.; W. R. LASTER, B. J. ABBOTT, J. VENDITTY & M. BARATTA: Antitumor activity of macromomycin B (NSC-170105) against murine leukemias, melanoma and lung carcinoma. Cancer Res. 35: 939~945, 1975
4) KUNIMOTO, T.; M. HORI & H. UMEZAWA: Reversible binding of macromomycin, a macromolecular peptide antibiotic, to cell membranes. J. Antibiotics 24: 203~205, 1971
5) KUNIMOTO, T.; M. HORI & H. UMEZAWA: Macromomycin, an inihbitor of the membrane function of tumor cells. Cancer Res. 32: 1251~1256, 1972
6) LIPPMAN, M. M.: *In vitro* studies on macromomycin (NCS-170105). Effects on cultured TA3Ha and L1210 cells. Cancer Chemoth. Rept. 58: 181~187, 1974
7) WINKELHAKE, J. L. & F. L. A. BUCKIRE: Radioimmune assay and characteristics of antibodies to macromomycin (NCS 170105). Cancer Res. 37: 1197~1204, 1977
8) SUZUKI, H.; T. NISHIMURA, K. MUTO & N. TANAKA: Mechanism of action of macromomycin: DNA strand scission, inhibition of DNA synthesis and mitosis. J. Antibiotics 31: 875~883, 1978
9) YAMASHITA, T.; N. NAOI, T. HIDAKA, K. WATANABE, Y. KUMADA, T. TAKEUCHI & H. UMEZAWA: Studies on auromomycin. J. Antibiotics 32: 330~339, 1979
10) OVEJERA, A. A.; R. K. JOHNSON & A. GOLDIN: Growth characteristics and chemotherapeutic response of intravenously implanted LEWIS lung carcinoma. Cancer Chemoth. Rep., Part 2, 5: 111~125, 1975
11) IM, W. B.; C. CHIANG & R. MONTGOMERY: Studies on macromomycin, an antitumor protein. J. Biol. Chem. 253: 3259~3264, 1978
12) ISHIDA, N.; K. MIYAZAKI, K. KUMAGAI & M. RIKIMARU: Neocarzinostatin, an antitumor antibiotic of high molecular weight: Isolation, physicochemical properties and biological activities. J. Antibiotics 18: 68~76, 1965
13) MEINHOFER, J.; H. MAEDA, C. B. GLASER, J. CZOMBOS & K. KUROMIZU: Primary structure of neocarzinostatin, an antitumor protein. Science 178: 875~876, 1972
14) FUJITA, H.; N. NAKAYAMA, T. SAWABE & K. KIMURA: *In vitro* distribution and inactivation of neocarzinostatin. Jap. J. Antibiotics 23: 471~478 ,1970

15) MAEDA, H. & J. TAKESHITA: Degradation of neocarzinostatin by blood sera *in vitro* and its inhibition by diisopropyl fluorosphate and N-ethylmaleimide. Gann 66: 523~527, 1975
16) MAEDA, H. & J. TAKESHITA: Inhibitors of proteolytic enzymes present the inactivation by blood of the protein antibiotic neocarzinostatin and its succinyl derivative. J. Antibiotics 29: 111~112, 1976