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Antitumor Activity of Cytotropic Heterogeneous Molecular Lipids (CHML) on Human Breast Cancer Xenograft in Nude Mice

QIMIN ZHAN[1], SHI-CHENG ZHAO[2] and ZHENG XU[3]

[1]*Department of Radiation Oncology, Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213;* [2]*Molecular Pharmacology, Memorial Sloan-Kettering Cancer Center, NY 10021;* [3]*Glory F & D Co. Ltd, Arlington, VA 22203, U.S.A.*

Abstract. *Cytotropic heterogeneous molecular lipid (CHML), which is a new anticancer agent with US patent number 5,260,067, has recently been shown to suppress tumor cell growth in multiple tumor lines and induce apoptosis in vitro (1). These results indicate that CHML may be an effective antitumor agent. In the present study, using both local injection and intravenous injection, we have investigated the suppressive effect of CHML on human breast caner cells MCF-7 xenograft in nude mice. In the local injection, CHML was introduced into nude mice implanted with human breast cancer xenograft at doses of 25 mg/tumor area (cm^2), 35 mg/tumor area (cm^2), or 50 mg/tumor area (cm^2), once every two days, total 3 times. The inhibition of tumor growth was 81.3%, 93.8% and 100%, respectively. In the intravenous injection, the nude mice bearing MCF-7 xenografts were treated with CHML at 10 mg/kg/day, or 15 mg/kg/day, or 20 mg/kg/day, once a day, total 7 days, the growth inhibition of tumor area was 58.1%, 77.4%, and 83.9%, respectively. At the same time, the toxicity of CHML was determined through examining the number of the white blood cell (WBC) and the activity of the serum glutamic-pyruvic transaminase (SGPT). However, no evident alterations of WBC and SGPT were detected in all animals treated with CHML, suggesting that CHML has little toxicity on nude mice. Taken together, these results indicate that CHML is an effective agent that suppresses breast tumor growth and suggest the possibility of using CHML in the clinical trial in the near future.*

At the present time, there is an increasing need to develop effective anticancer agents. The usage of the current antitumor drugs in clinic has greatly been compromised by their side effects. Therefore, the ideal anticancer agents would be expected to specifically kill tumor cells but have little cytotoxicity on normal tissues and organs. CHML (Cytotropic Heterogeneous Molecular Lipid) was extracted from the natural product and developed by Glory F & D Co. Ltd in USA. In our previous report, CHML has been shown to strongly inhibit tumor cell growth in multiple lines using a typical colony survival assay. In a treatment at concentration of 50 μg/ml for 6 hours, CHML is able to suppress 50% of the tumor cell colony formation. At a concentration of 100 μg/ml, more than 90% of the cells were killed in human breast cancer MCF-7 and human colorectal carcinoma lines. In contrast, growth suppression of non-cancerous human skin fibroblasts by CHML was observed much less than that seen in tumor lines, indicating that CHML is an efficient inhibiting agent in tumor cells growth and is able to generate greater suppression in tumor than in noncancerous cells. With the use of DNA fragmentation assay, CHML was found to induce apoptosis in both MCF-7 and RKO cells following treatment at a concentration of 75 μg/ml for 8 hours. Interestingly, the tumor suppressor p53 protein was found to elevate in RKO cells after cells were treated with CHML. Consistent with this finding, Bax, which is regulated by p53 and is able to promote apoptosis, was also found to increase in the same kinetic manner as p53 (1). Clearly, those results suggest that CHML might be a potent tumor-inhibiting agent. Recently, the suppressive effect of CHML on human breast cancer xenograft in nude mice has been investigated. We have found that CHML is able to strongly inhibit growth of breast cancer MCF-7 xenograft implanted in the animals, but does not generate evident toxicity to the animals, as measure by the number of white blood cells (WBC) and the activity of the serum glutamic-pyruvic transaminase (SGPT). These results indicate that CHML is a good candidate in the future clinical trial.

Correspondence to: Qimin Zhan.

Key Words: CHML, breast cancer, tumor suppression.

Materials and Methods

Drug. CHML was supplied by Glory F & D Co. Ltd in USA. CHML consists of 1% squalene, 80% unsaturated fatty acids, 15% saturated fatty acids and 4% liposoluble vitamins. All components of CHML were extracted from natural products and prepared by the lipid-activated methods.

Cells and animals. The human breast cancer MCF-7 cells were described previously (1) and used for generating xenograft. Six-week-old Athymic nu/nu BALB/c mice (both female and male), which were purchased from the Charles River Laboratories, Wilmington, MA, were used in this study. The mice were maintained in a specific pathogen-free environment in compliance with United States Public Health Service guidelines governing the care and the maintenance of experimental animals. All animal studies were conducted in accordance with the principles and the procedures outlined in the NIH "Guide for the Care and Use of Laboratory Animals". Mice were fed with an autoclaved laboratory rodent diet (Tecklad LM-485; Western Research Products, Orange, CA).

Animal treatment with CHML. Animals were challenged with 0.5 ml of a single-cell suspension containing 5 X 10^6 MCF-7 cell by subcutaneous injection in the right flank. 12 days after tumor cell inoculation, animals were treated with CHML via either local injection or intravenous injection. At the local injection, nude mice were treated with CHML at 25 mg/tumor area (cm^2), 35 mg/tumor area (cm^2), or 50 mg/tumor area (cm^2). Treatment was applied once every two days, total 3 times. At intravenous injection, nude mice were treated with CHML at 10 mg/kg/day, or 15 mg/kg/day, or 20 mg/kg/day, once each day, total 7 days.

Tumor measurements. Tumor measurements were performed weekly using the Vernier calipers. Tumor area was calculated using the formula: length/2 X width/2 X JI. Mean tumor area was plotted against time in weeks to monitor tumor growth.

Determination of toxicity. The numbers of the White Blood Cells (WBC) were examined weekly by traditional direct counting with hemocytometer. The activity of the serum glutamic-pyruvic transaminase (SGPT) was also measured using a standard procedure at the same time.

Table I. *Effect of CHML on human breast cancer xenograft in nude mice.*

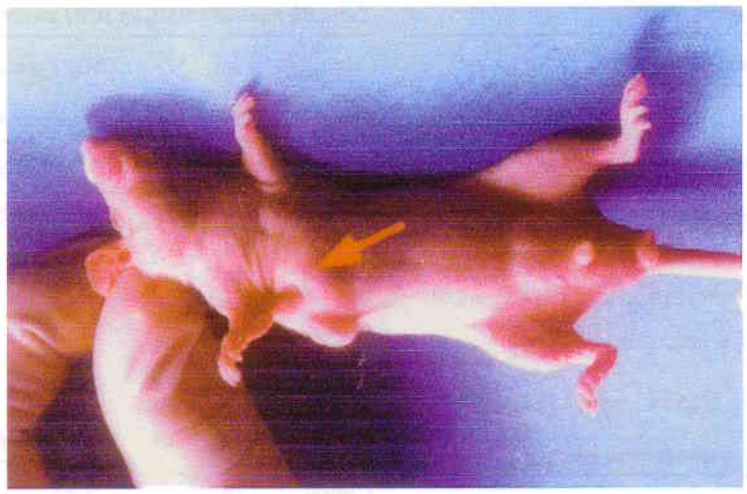
		Local injection			
Group	No. of animals	Dose/cm^2	Tumor size (cm^2)	Inhibition (%)	P
Control	10	-	3.2±0.4		
Group 1	10	25 mg	0.6±0.04	81.3	<0.01
Group 2	10	35 mg	0.2±0.02	93.8	<0.01
Group 3	10	50 mg	0	100	<0.01
		Intravenous injection			
Group	No. of animals	Dose/kg	Tumor size (cm^2)	Inhibition (%)	P
Control	10	-	3.1±0.6		
Group 1	10	10 mg	1.3±0.05	58.1	<0.01
Group 2	10	15 mg	0.7±0.07	77.4	<0.01
Group 3	10	20 mg	0.5+0.04	83.9	<0.01

Human breast cancer MCF-7 cells (5×10^6) were inoculated in the right flank of nude mice. 12 days after inoculation, the animals were treated with CHML via either local injection or intravenous injection at the indicated doses for total three weeks (see Materials and Methods). The tumor size was measured to evaluate the growth inhibition of MCF-7 xenograft by CHML. The results presented here is collected and analyzed three weeks following treatment with CHML.

Results

We have previously reported that CHML is able to suppress tumor cell growth and to induce apoptosis in multiple tumor lines *in vitro.* To further determine the growth-suppressive effect of CHML in tumor cells *in vivo,* we have inoculated the human breast cancer MCF-7 cells in nude mice and then investigated the growth suppression of xenografts by CHML. To do this, 0.5 ml of single cell-suspension of human breast cancer MCF-7 cells was inoculated by subcutaneous implantation in the right flank of nude mice. 12 days following inoculation, the animals were subject to treatment with CHML through either local injection or intravenous injection. In the experiments of local injection, animals were divided into four groups, including control group, which was treated with saline and three CHML-treated groups, which were exposed to different concentrations of CHML. The experiments were repeated for three times and totally, forty nude mice were used in the study. In CHML-treated groups, three difference dosages were applied. They were 25 mg/tumor area (cm^2), 35 mg/tumor area (cm^2) and 50 mg/tumor area (cm^2), respectively. Each animal was treated once every two days for a total of three times. The tumor size was measured each week for three weeks. In Figure 1, nude mouse harboring human breast cancer xenograft was treated with CHML at a dose of 35 mg/tumor area (cm^2). Two weeks later, most of the tumor area was shown to be necrotic, indicating that CHML is able to cause tumor cell death. The results of the entire experiments were summarized in Table I and Figure 2. After three weeks, the growth inhibition, in contrast to control group treated with saline, was evidently seen in each CHML-treated group. It appears that inhibition of tumor growth is dose-dependent. A dose of 25 mg/tumor area (cm2) resulted in 81.3% growth inhibition of the tumor xenograft, while CHML at 35 mg/tumor area (cm^2) inhibited 93.8% tumor growth. Interestingly, a dose of 50 mg/tumor area (cm^2) was shown to generate 100% growth suppression of the xenograft in nude mice.

Next, we have further determined the treatment efficacy of CHML in tumor xenograft in nude mice via the intravenous injection. After tumor cells inoculated in nude mice, the animals with MCF-7 xenografts were treated with CHML at different does. As shown in Table I, a dose of CHML at 10 mg/kg/day suppressed 58.1% tumor growth. Similar to the local injection, higher doses produced



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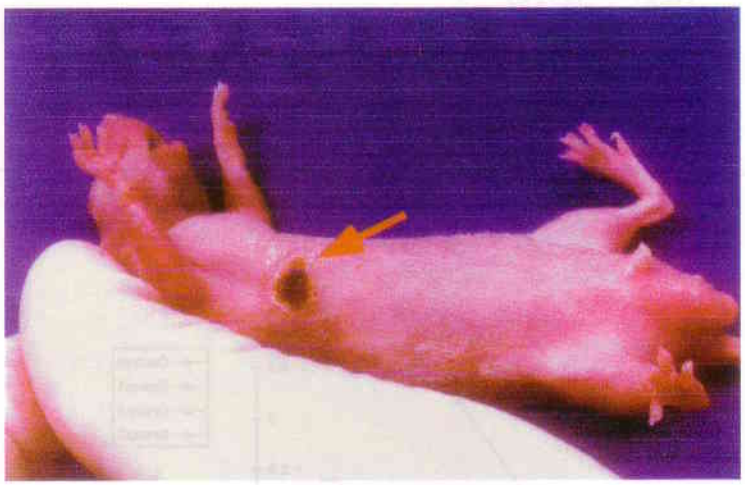

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Figure 1. *Effect of CHML on human breast cancer MCF-7 xenograft in nude mice. Human breast cancer MCF-7 cells (5 X 10^6) in single-cell suspension were inoculated in the right flank of nude mice. 12 days later, nude mice were treated with CHML by local injection at a concentration of 35 mg/tumor area (cm^2). Treatment was applied once every two days, total 3 times. The CHML-induced growth suppression of the breast cancer xenografts in nude mice was evaluated via the measurement of the tumor size at three weeks posttreatment. The representative result is shown in Figure 1.*

stronger inhibition of tumor growth. CHML at 20 mg/kg/day inhibited 77.4% tumor growth and 20 mg/kg/day suppressed 83.9% tumor growth, as measured by the size of tumor area.

To determine if CHML generates any toxicity during the treatment in nude mice, we examined the number of white blood cells (WBC) and the activity of serum glutamic-pyruvic transaminase (SGPT), which are usually considered as two of most important criteria to evaluate cytotoxicity of new anti-cancer agents. The results were summarized in Table II and Table III. Evidently, nude mice treated with CHML at all doses did not exhibit significant alterations of the numbers of WBC and the activity of SGPT in contrast to the control group. These results indicate that the treatment of CHML at such a dose range is of little toxicity in nude mice.

Discussion

For many years, local treatment of breast cancer was considered the domain of the surgeon. Over the last 20 years, the approach to the local treatment of breast cancer has radically changed due to our increased understanding of the biology of the disease, detection of small tumors, increased emphasis on systemic therapy, and greater patient involvement in the decision-making process. Today, local treatment of breast cancer involves the collaborative surgeons, radiologists, pathologists, radiation oncologists, reconstructive surgeons, and medical oncologists working with the patients (2). A large body of experience on the use of preoperative chemotherapy has accumulated since the its first use in the mid 1960's (3-5). Clinical trials and experience have confirmed that a combined modality

Table II. *Effect of CHML in the numbers of WBC.*

Group	No. of animals	WBC Numbers (× 1000/mm^3) 1 week	2 weeks	3 weeks	P
		Local injection			
Control	10	14±2.5	13±4	13.5±4.5	-
Group 1	10	13.5±2.5	12±3.5	13±4	>0.05
Group 2	10	15.5±2	14.5±3	14±3.5	>0.05
Group 3	10	15±3.5	12.5±4	13.5±4	>0.05
		Intravenous injection			
Control	10	15±2	14±2.5	15.5±4.5	-
Group 1	10	12.5±3.5	13±4	13.5±3.5	>0.05
Group 2	10	13.5±3	14±4	15±2.5	>0.05
Group 3	10	14.5±4	13±3.5	14±4	>0.05

Table III. *Effect of CHML on SGPT activity.*

Group	No. of animals	SGPT activity (unit/L) Before treatment	After treatment	P
		Local injection		
Control	10	19±3	17±5	-
Group 1	10	17±6	16±7	>0.05
Group 2	10	21±4	22±5	>0.05
Group 3	10	18±7	16±9	>0.05
		Intravenous injection		
Control	10	20±5	21±7	
Group 1	10	22±7	19±9	>0.05
Group 2	10	18±6	19±8	>0.05
Group 3	10	23±8	21±6	>0.05

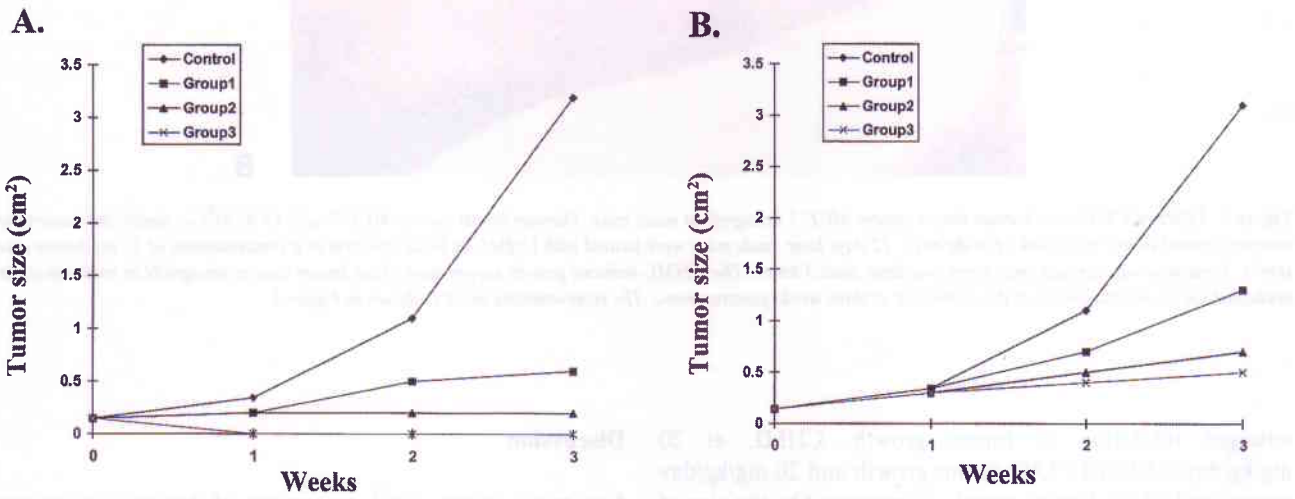

Figure 2. *Growth suppression of human breast cancer MCF-7 xenograft in nude mice by CHML. (A). Local injection: Total forty nude mice used in this experiment were divided into four groups. 12 days following tumor cells inoculation, the control group was treated with 0.5 ml of saline but other groups were treated with CHML at different concentrations. Usages of the CHML dose were 25 mg/tumor area (cm^2) for group1, 35 mg/tumor area (cm^2) for group 2 and 50 mg/tumor area (cm2) for group 3. The treatment was applied once every two days, total three times. Measurement of the tumor size was carried out each week. (B). Intravenous injection: After tumor cells inoculation, the animals were treated with CHML through the intravenous injection. Usages of the CHML dose were 10 mg/kg/day (group1), 15 mg/kg/day (group 2) and 20 mg/kg/day (group 3), respectively. The tumor size was measured using the Vernier calipers.*

approach is the most effective way to achieve local treatment while providing the advantages of systemic adjuvant treatment (6-7).

Problems still exist in clinical trials for breast cancer. Up to 30% of early stage breast cancer patients have cancer cells in the blood and bone marrow at the time of surgery as detected by immunohistochemistry or reverse PCR (8-9). This indicates the presence of distant micrometastases at the time of diagnosis in most locally advanced cancer patients (10). Most patients with local advanced tumors fail

to achieve local control following systemic chemotherapy due to drug resistance (11) or induced resistance of cancer cells (12). However, the local treatment with chemoagents still remains doubt because of the limited efficacy and the tumor reoccurrence. In contrast, the systemic therapy often displays adverse effect or toxicity although it may efficiently eliminate tumors (13-21). Therefore, explorations of new ideal anticancer agents that are able to specifically kill tumor cells but have little cytotoxicity on normal tissues and organs are currently the main task for scientists in the field of cancer therapy.

In the present study, we have found that Cytotropic heterogeneous molecular lipid (CHML), which is a new anticancer agent with US patent number 5,260,067, can strongly suppress human breast cancer MCF-1 xenograft in nude mice via either local treatment or intravenous injection. However, CHML treatment in nude mice did not present any significant toxicity, as measured by the numbers of white blood cells and the activity of activity of serum glutamic-pyruvic transaminase (SGPT).

Recently, we have reported that using colony formation assay, CHML has been shown to strongly suppress tumor cell growth in multiple cell lines. In treatment at a concentration of 50 *ug*/ml for 6 hours, CHML is able to suppress 50% of the tumor cell colony formation. At a concentration of 100 *ug*/ml, more than 90% of the cells were killed in human breast carcinoma MCF-7, colorectal carcinoma RKO, kidney carcinoma G410, lung carcinoma H1299 and human myeloid leukemia ML-1 lines. With regard to the mechanism by which CHML suppresses cell growth, we have observed that CHML is able to induce apoptosis in tumor cells, including MCF-7, ML-1, H1299 and RKO after treatment at a concentration of 75 *ug*/ml for 8 hours. Interestingly, the tumor suppressor p53 protein elevated in RKO cells at post-treatment. In addition, the p53-regulated Bax gene that promotes apoptosis is increased following CHML treatment, indicating that CHML-induced growth suppression may involve the p53-regulated pathway (1).

Currently, a clinical trial with this CHML is being conducted in China. Based on the preliminary results, CHML has exhibited an effective treatment to multiple types of human tumors, but no significant cytotoxicity was observed at the doses of clinic application (Zheng Xu *et al*, manuscript in preparation). In the present study, we have shown that CHML can greatly suppress human breast cancer xenograft in nude mice by both local and systemic treatments, indicating that CHML is a potentially strong candidate of new anticancer drugs.

With regard to the toxicity of CHML in the treatment of human breast cancer xenograft on nude mice, there are no any evident alterations in the numbers of the white blood cell (WBC) and the activity serum glutamic-pyruvic transaminase (SGPT) at the doses, which are used to generate suppressive effect on xenografts. Therefore, these results indicate that CHML is a safe anticancer agent in such a dose range. However, future investigation will be carried out to provide more information on the cytotoxicity of CHML, including that in both acute and chronic phases.

In summary, new anticancer agent CHML has been shown to substantially suppress human breast cancer xenograft in nude mice via local injection and intravenous injection, but exhibits little cytotoxicity to the mice, as determined by measurement of the white blood cells and the activity serum glutamic-pyruvic transaminase (SGPT). These results have provided the strong information to guide the clinic trial in the near future.

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