

## CHML Suppresses Cell Growth and Induces Apoptosis in Multiple Human Tumor Lines

QIMIN ZHAN<sup>1</sup> and ZHENG XU<sup>2</sup>

<sup>1</sup>Department of Radiation Oncology, Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213;

<sup>2</sup>Glory F & D Co. Ltd, Arlington, VA 22203, U.S.A.

**Abstract.** In the present study, we have investigated the effect of cytotoxic heterogeneous molecular lipid (CHML), a new anticancer agent, on growth suppression in a variety of human tumor cell lines. At a non-toxic concentration (a range from 25 µg/ml to 100 µg/ml), CHML has shown to strongly inhibit tumor cell growth by using a typical colony survival assay. At a treatment of 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 (the therapeutic dosage in the clinical trial), more than 90% of the cells were killed in human breast carcinoma MCF-7, colorectal carcinoma RKO, kidney carcinoma G410, lung carcinoma and human myeloid leukemia ML-1 lines. In contrast, growth suppression of non-cancerous human skin fibroblasts by CHML was observed much less than that seen in tumor lines. These results indicate that CHML is an efficient inhibiting agent in tumor cell growth and is able to generate greater suppression in tumor cells than in noncancerous cells. With the use of DNA fragmentation assay, CHML was found to induce apoptosis in MCF-7, ML-1, H1299 and RKO lines after treatment at a concentration of 75 µg/ml for 8 hours. Following the CHML treatment, the tumor suppressor p53 protein elevated in RKO cells at 2 h posttreatment. The induction of p53 reached a peak at 4 hr and returned to normal level 16 hr later. Consistent with this result, Bax, which is regulated by p53 and is able to promote apoptosis, was also found to increase in a same kinetic manner as p53. These results suggest that the p53-pathway is activated by CHML and the activation of p53 may contribute to CHML-induced apoptosis in some tumor cells, such as MCF-7, RKO and ML-1. Considering that CHML is able to induce apoptosis in H1299 cells, which are of p53-negative status, it is speculated that CHML induces programmed cell death through both the p53-dependent and-independent pathways.

Apoptosis is a genetically encoded cell death program defined by characteristic morphologic and biochemical changes (1). Multiple factors modulate apoptosis induction, including growth factors, intracellular mediators of signal transduction, nuclear protein regulating gene expression, DNA replication and cell cycle (2). Apoptosis is also a major mechanism by which chemotherapeutic agents and ionizing radiation kill cells (3). Among the numerous factors known to modulate cancer-related apoptosis, p53 and Bcl-2 are most extensively characterized mechanistically. p53 can activate an apoptosis response to a variety of DNA damaging agents in addition to its implication in the control of cell cycle checkpoints (4-7). Therefore, p53 can alter the cellular responses to DNA damage in different ways. It has been well accepted that the biological functions of p53 are at least in part mediated through its down stream genes such as p21/WAF1(8), Gadd45 (4,9) and Bax (10-11). P21/WAF1 is a potent inhibitor of multiple cell cycle dependent kinases and plays an important role in the G1 checkpoint after DNA damage (12-14). Gadd45 has recently been found to interact with Cdc2 kinase and this interaction causes the dissociation of Cdc2/cyclin B1 complex, yet leads to the inhibition of Cdc2 kinase activity (15). Bax is a member of Bcl2 family and promotes apoptosis (16-17). The induction of Bax following ionizing radiation depends on the normal cellular p53 function (10). Bcl-2 is activated by chromosomal translocation in follicular lymphoma and demonstrates a profound capacity to block apoptosis (18-19). Exogenous expression of Bcl-2 has been shown to protect cells from apoptosis induced by growth factor withdrawal, ionizing radiation and chemotherapeutic agents (20-21). Also, Bcl-2 has been found to be capable of modulating apoptosis caused by the expression of p53 protein(22-23). Interestingly, in the cellular responses to genotoxic stress, activation of p53 was shown to simultaneously down-regulate Bcl-2 and up-regulate Bax (10-11, 24,25). Both Bcl-2 and Bax can form homodimers or heterodimers, and a model has been proposed in which the ratio of Bcl-2 to Bax determines survival following a stimulus (17). However, apoptosis is complex because there are p53-independent pathways involved in the control of apoptosis after certain stimuli, such as glucocorticoid treatment, calcium-dependent activation and *in vitro* aging (5).

Correspondence to: Qimin Zhan, Department of Radiation Oncology, Cancer Institute University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA.

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CHML (Cytotropic heterogeneous Molecular Lipids) is a new anticancer drug developed by Glory F & D Co. Ltd, USA. The patent of the drug has been granted under U.S. patent number 5,260,067. In the clinical trials currently carried out in P.R. China, Mexico and some countries of Southeast Asia, CHML has manifested an effective treatment for multiple types of human tumors (Zheng Xu *et al*, manuscript in preparation). To gain some insight in the molecular mechanism by which CHML functions as a potent and effective anticancer agent, a series of experiments has been conducted recently by means of cellular and molecular approaches. Here we report that CHML is able to strongly suppress cell growth as measured by clonogenic survival assay. Interestingly, the magnitude of growth suppression generated by CHML was greater in cancer cell lines (RKO, H1299, ML-1, G410 and MCF-7) compared with non-cancerous cell lines (AG1522 and GM536). Following the treatment with CHML, a rapid and strong apoptosis, as detected by DNA fragmentation, was observed in multiple cell lines including both wild-type p53 cell lines and cells with negative p53 status. Like many other therapeutic agents, CHML was found to increase the protein levels of tumor suppressor p53, as well as its downstream effector genes Bax and p21. These results suggest that the inhibitory effect of CHML on tumor cells may be mediated through the process of apoptotic cell death and that CHML-induced apoptosis involves both p53-dependent and -independent pathways.

## Materials and Methods

**Drug.** A new anticancer agent, cytotropic heterogeneous molecular lipid (CHML) was developed and provided by Zheng Xu. The patent of the drug has been granted under U.S. patent number 5,260,067. CHML consists of 1% squalene, 80% unsaturated fatty acids, 15% saturated fatty acid and 4% liposoluble vitamins. All components of CHML were extracted from nature and prepared by lipid-activated methods.

**Cell lines and culture conditions.** The human myeloid leukemia ML-1, human breast carcinoma MCF-7, human lung carcinoma H1299, human colorectal carcinoma RKO lines, human kidney carcinoma G410, human skin fibroblast AG155 and human lymphoblast GM536 were grown in RPMI 1640 medium with 10% fetal calf serum as described previously (4, 9, 26).

**Clonogenic survival assay.** 600 growing cells were seeded in 100-mm-diameter dishes, with 3 plates per point in each experiment. 20 hr later, indicated doses of CHML were added to the cell culture. Following incubation at the indicated time, the medium was removed and the plates were washed once with PBS, then fresh medium containing no CHML was added to the plates. The cells were fixed at 14 days and scored for colonies containing at least 50 cells (26).

**Microtiter survival assay.** A microtiter clonogenic survival assay suitable to suspension growing cells was carried out in a survival study. Briefly, the human lymphoblastoid GM536 cells and human myeloid leukemia ML-1 cells were treated with the indicated doses of CHML and then serially diluted in half-log steps using complete medium as diluent. 200  $\mu$ l of the selected diluted cell suspension were placed in each of the 24 flat bottom wells of a covered 96 well microtiter plate (Costar Corporation). 3 dilutions (20 cells per well, 66 cells per well and 200 cells per well) were

used per plate. The plates were placed in the incubator containing 5% CO<sub>2</sub>. At 10-14 day intervals, the wells were examined with microscope (Bunton Instrument Company) for cell growth and scored as positive or negative. Finally, survival was calculated with the Poisson formula on the basis of the fraction of wells in which cells were not proliferating (9, 27).

**Western immunoblotting analysis of p53, Bax and p21 proteins.** For measurement of p53, Bax and p21 protein levels, total cellular protein was prepared as previously stated (28). 100  $\mu$ g of the protein was loaded onto 12% SDS-polyacrylamide gels. Following electrophoresis, the protein was transferred electrophoretically to Immobilon membranes (Millipore, Bedford, MA). The membranes were then blocked for 30 min in 5% nonfat milk at room temperature. A monoclonal mouse antibody to p53 (pAB1801, Oncogene Science, NY) was used to measure p53 protein levels and a polyclonal antibody to Bax (Santa Cruz, CA) was used in the experiment. For p21 detection, the membrane was probed with polyclonal rabbit anti-human-p21 antibody (Pharmingen, CA). Antibody reaction was with a chemiluminescence detection procedure according to the manufacture's recommendation (Amersham).

**DNA fragmentation assays.** In agarose gel electrophoresis assays, DNA was extracted from the cells 8 hr after the cells were treated with CHML at the indicated concentration. The cells were lysed in a buffer of 10 mM Tris and 1 mM EDTA (TE buffer, pH 8.0) containing 0.2% Triton X-100, 0.1 mg/ml proteinase K and 0.1 mg/ml RNase A. The lysate was extracted with phenol/chloroform, ethanol precipitated and dissolved in TE buffer. DNA was electrophoresed in 1.2% agarose gels containing ethidium bromide (10, 29).

## Results

**Suppression of cell growth by CHML.** To investigate the effect of CHML on tumor cells, a growth suppression assay via colony formation was conducted. Cells from different human lines were seeded at a density of 600 cells per 100 mm dish. The indicated amounts of CHML were added to cell cultures. Following incubation for 6 hours, the medium was removed and fresh medium was added. 14 days later, the cells were fixed and scored for colonies. As shown in Figure 1 and Table I, CHML was able to reduce the yield of colonies in all human tumor lines tested. At a treatment of concentration of 50  $\mu$ g/ml for 6 hr, CHML was able to suppress 30% to 50% colony formation of tumor cells and 75  $\mu$ g/ml of CHML was seen to generate 70% to 80% growth suppression. At a concentration of 100  $\mu$ g/ml, which is a therapeutic dosage in the clinical trial, more than 90% of cells were killed in human breast carcinoma MCF-7, colorectal carcinoma and kidney carcinoma lines. Apparently, the growth suppression of tumor cells is manifested in a dose-dependent manner. In the case of human myeloid leukemia ML-1 cells, a suspension-growing line, a microtiter clonogenic survival assay was carried out. In this experiment, CHML, at the indicated concentrations, was constantly incubated with the cell cultures for 14 days (see Materials and Methods). In Table I and Figure 2, 25  $\mu$ g/ml of CHML was able to suppress 90% of cell growth. Only 3% survival of ML-1 cells was observed at a CHML concentration of 50  $\mu$ g/ml. These results indicate that CHML may be a potent anticancer agent because of its capability of growth suppression in multiple tumor lines.

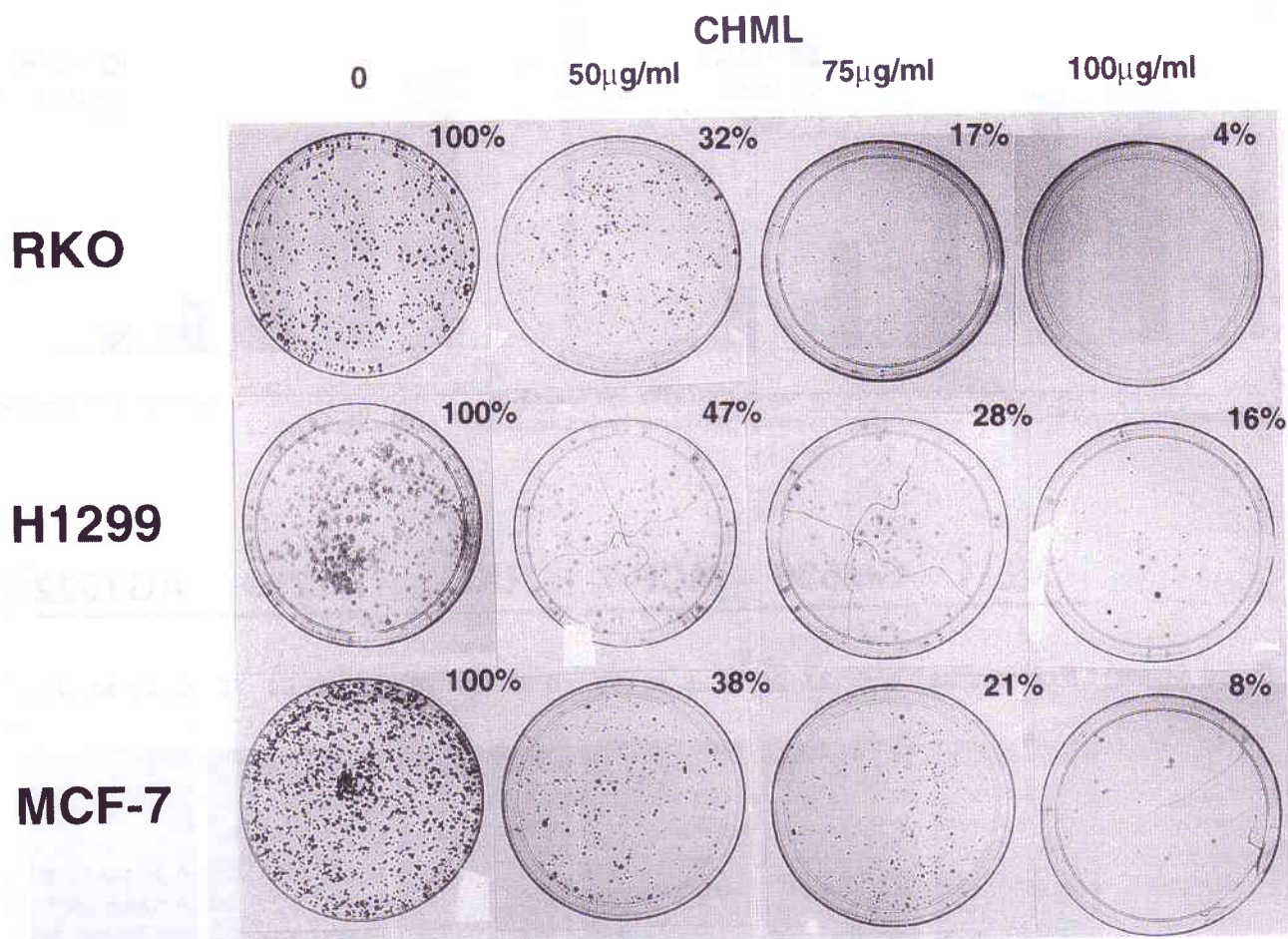


Figure 1. Suppression of cell growth with the treatment of CHML. Human H1299 (lung carcinoma), RKO (colorectal carcinoma) and MCF-7 (breast carcinoma) cells were treated with indicated doses of CHML for six hours. The medium was removed and plates were washed once with PBS, then fresh medium containing no CHML was added into plates. The cells were fixed at 14 days and scored for colonies containing at least 50 cells.

In order to determine the effect of CHML on non-cancerous types of cell lines, human skin fibroblasts AG1522 and normal lymphoblastoid MG536 cells were included in the experiments of growth suppression. As shown in Table I and Figure 2, CHML was shown to suppress the growth of these two non-cancerous lines. Interestingly, at the same concentration, CHML was able to generate more appreciable growth suppression in all tumor lines in contrast to that generated in human skin fibroblasts and normal lymphoblasts. For example, at the dosages of 25, 50, 75, and 100  $\mu$ g/ml of CHML, the survivals of RKO cells were 56%, 32%, 17%, and 4% but AG1522 showed a survival of 92%, 61%, 44% and 29% at each parallel concentration. The difference in growth suppression between AG1522 and RKO or MCF is statistically significant. Similarly, CHML was shown to produce a greater growth suppression in ML-1 cells than in MG536 line at the concentration of 12.5, 25 and 50  $\mu$ g/ml (Table I and Figure 2). Therefore, CHML may be more effective in the growth suppression of tumor cell lines than in that of non-cancerous cells.

*CHML causes apoptosis in multiple tumor cell lines.* Apoptosis is one of the major pathways that lead to cell killing following the treatment with multiple anti-tumor drugs (3, 30-31). To explore the mechanism by which CHML causes the suppression of tumor cell growth, cellular DNA fragmentation was detected in different cell lines after treatment with CHML. As illustrated in Figure 3, marked DNA fragmentation with nucleosome-size laddering was detected in all tumor lines (ML-1, RKO and H1299) after the cells were treated with CHML for 8 hours. Apparently, sensitivity to CHML treatment varied among these cell lines. In ML-1, evident apoptosis was observed at a concentration of 12.5  $\mu$ g/ml, but both RKO and H1299 required a higher dose (50  $\mu$ g/ml) of CHML for the onset of apoptosis. In the case of GM536, a normal lymphoblastoid line, DNA fragmentation was seen after 25  $\mu$ g/ml of CHML. In contrast, there was no evident DNA fragmentation seen in AG1522 treated with CHML, even at a concentration of 75  $\mu$ g/ml. These results indicate that the mechanism by which CHML suppresses cell growth may be mediated at least in part through apoptosis

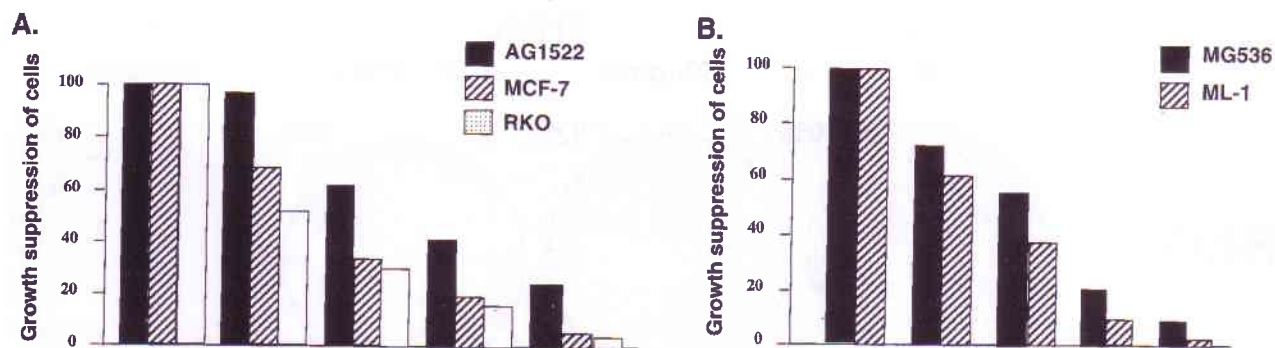


Figure 2. Sensitivity of human cancerous and non-cancerous cells to CHML. Human cells with cancerous or non-cancerous phenotypes were treated with CHML and growth suppression assay was performed as Figure 1. Quantitative analysis was evaluated as illustrated in Table I.

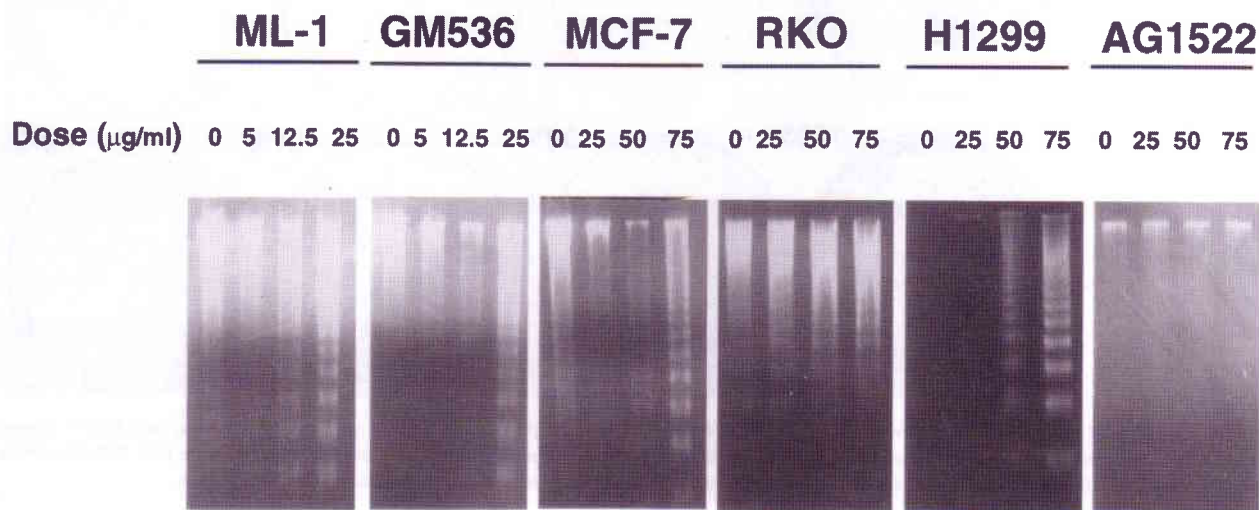


Figure 3. Apoptosis induced by the treatment of CHML in human cells. Human RKO, H1299, MCF-7, ML-1 and AG1522 cells were treated with the indicated concentration of CHML for 8 hours and cells were collected for the assay of cellular DNA fragmentation.

and that the onset of apoptosis in tumor cells is more susceptible to CHML treatment in contrast to non-cancerous cells.

*Induction of p53, p21 and Bax protein levels by CHML.*

Previous reports have demonstrated that the tumor suppressor p53 plays an important role in the cell cycle checkpoints and in the process of apoptosis induced by certain genotoxic agents (4-7, 32-33). In present study, the induction of p53 protein, as well as that of Bax and p21 protein, was measured by western immunoblotting analysis. As shown in Figure 4, the elevated p53 protein level was seen at 2 hr following the MCF-7 cells treated with CHML at a concentration of 50 μg/ml. The induction of p53 protein reached its peak at 4 hr posttreatment and returned to

normal level at 16 hr. Similarly, the protein level of Bax, a p53-regulated and apoptosis-associated gene (10-11, 17, 25), was induced in MCF-7 line after treatment with 50 μg/ml of CHML. The peak induction for Bax was observed at 4 hr and an elevated level remained till 16 hr. Interestingly, p21, a p53 downstream effector gene that plays a critical role in cell cycle G1 checkpoint (8, 12-13) was also induced by CHML in MCF-7 cells (Figure 4). These results suggest that CHML is able to activate the p53-pathway, which may function in CHML-induced apoptosis. However, CHML was also found to induce apoptosis in H1299 cells, which contain deleted p53 gene (Figure 3). Taken together, p53-dependent and -independent pathways are both involved in CHML-associated apoptosis.

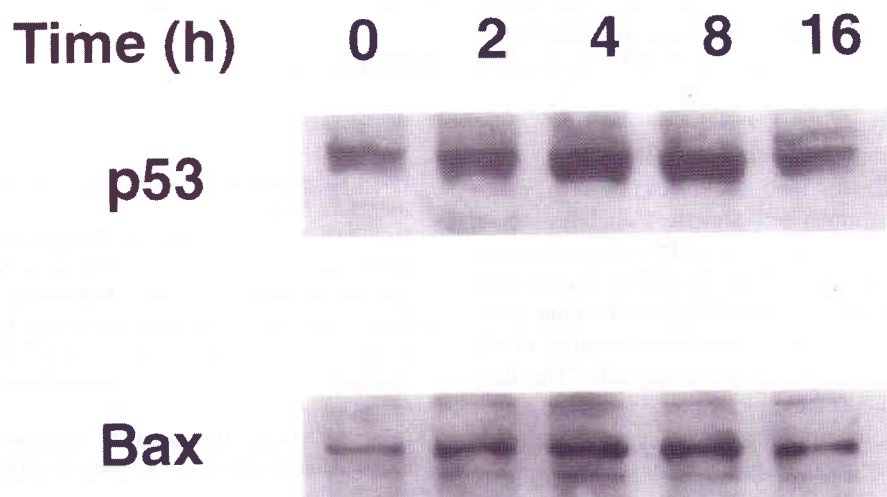


Figure 4. Immunoblotting analysis of p53, p21 and Bax in cells treated with CHML. Human colorectal carcinoma RKO cells were treated with CHML (75 mg/ml) and cells were collected at indicated time points for protein analysis.

Table I. Effect of CHML on the growth suppression of human cell lines.

Cell line	Cell type	CHML (Dose $\mu\text{g/ml}$ )				
		0	25	50	75	100
MCF-7	Human breast carcinoma	100 $\pm$ 7%	67 $\pm$ 4%	38 $\pm$ 3%	21 $\pm$ 1.5%	8 $\pm$ 0.6%
RKO	Human colorectal carcinoma	100 $\pm$ 6%	56 $\pm$ 3%	32 $\pm$ 4%	17 $\pm$ 1.4%	4 $\pm$ 0.5%
H1299	Human lung carcinoma	100 $\pm$ 9%	72 $\pm$ 6%	47 $\pm$ 4%	28 $\pm$ 2%	16 $\pm$ 1.9%
G410	Human kidney carcinoma	100 $\pm$ 6%	68 $\pm$ 4%	41 $\pm$ 5%	26 $\pm$ 1.6%	10 $\pm$ 1.1%
AG1522	Human skin fibroblast	100 $\pm$ 10%	92 $\pm$ 7%	61 $\pm$ 6%	44 $\pm$ 2.8%	29 $\pm$ 3%
		0	5	12.5	25	50
MIL-1	Myeloid leukemia	100 $\pm$ 7%	61.8 $\pm$ 6%	38.3 $\pm$ 3%	9.1 $\pm$ 0.7%	2.7 $\pm$ 0.1%
MG536	Normal lymphoblast	100 $\pm$ 8%	79.4 $\pm$ 4%	54.5 $\pm$ 3%	21.5 $\pm$ 2%	9.4 $\pm$ 0.4%

## Discussion

The results presented here demonstrate that a new anticancer agent, cytotoxic heterogeneous molecular lipid (CHML) is able to strongly suppress tumor cell growth. After treatment with a concentration of 50  $\mu\text{g/ml}$  for 6 hr, CHML was found to reduce more than 50% of tumor cell colony formation in RKO, MCF-7, G410, H1299 and ML-1 cells derived from different tissues including colorectal carcinoma, breast cancer, kidney carcinoma, lung carcinoma and myeloid leukemia. At a concentration of 100  $\mu\text{g/ml}$ , the dose in the

clinical trial, more than 90% of tumor cells were killed. In contrast, noncancerous cells, such as skin fibroblasts and normal lymphoblasts, were less sensitive to the treatment of CHML at the same concentration. Using cellular DNA fragmentation assay, CHML showed a strong capability to induce apoptosis in multiple tumor cell lines including RKO, H1299 and ML-1 but not skin fibroblasts. Interestingly, the induction of tumor suppressor p53, which plays an important role in apoptosis induced by certain genotoxic agents, was observed in RKO cells treated with CHML. In addition, a p53 regulated and apoptosis associated protein Bax was also

found to increase following the treatment with CHML. Taken together, CHML is a potent growth inhibitor of tumor cells. Its growth inhibitory function may be mediated at least in part through the initiation of apoptotic process.

The observations reported in this paper provide striking information concerning the ability of CHML as a newly developed anticancer drug and the mechanism by which CHML suppress the tumor cell growth. Compared to its strong inhibition in tumor cell growth, CHML showed a weak inhibitory effect on non-cancerous cells such as human skin fibroblasts and human normal lymphoblasts at the same dose, suggesting that the tumor cells may be more sensitive to the treatment of CHML than the non-cancerous cells. This data is reflected by the manifestation of little side effect in the CHML clinical trial compared to some other existing clinical anticancer drugs (Zheng Xu, in preparation). However, the precise mechanism for this selectivity is currently unclear. It might be speculated that alterations in the membrane of cancer cells facilitate the uptake of CHML into cells, since this anticancer agent is a cytotoxic heterogeneous molecular lipid.

It has been well accepted that Programmed Cell Death (apoptosis) is a predominant biological event leading to cell killing following treatment with anticancer drugs (3, 14, 30-31). Taxol, which stabilizes microtubulin polymerization, is found to activate apoptotic cell death (33). In this study, CHML has shown to strongly induce apoptosis in a variety of tumor cell lines, suggesting that apoptotic death is one of the major mechanisms by which CHML functions as a potent anticancer drug. The tumor suppressor p53 is a critical protein involved in cellular responses to genotoxic stress, including ionizing radiation and chemotherapeutic agents (4-7, 28, 32). Functional p53 is required for ionizing radiation-induced apoptosis (34). Loss of functional p53 will alter the sensitivity of tumor cells to certain therapeutic agents. Disruption of p53 is found to sensitize tumor cells to taxol and cisplatin (33, 35). Our finding that CHML increases the protein level of p53 demonstrates that p53 may be involved in the apoptosis induced by CHML in cells containing wild-type p53. Consistent with this observation, Bax protein level is also found to elevate following CHML treatment. Since Bax protein is regulated by p53 and able to promote apoptosis (10-11, 17, 25), the activation of the p53-pathway evidently contributes to the CHML-induced apoptosis. However, CHML-activated apoptosis is complex. H1299 that contains negative-p53 status is able to undergo apoptosis after treatment with CHML. Since the initial target of CHML is the cell membrane, it is very possible that CHML is capable of activating other p53-independent pathways, yet lead to apoptosis. In summary, CHML suppresses tumor cell growth through both p53-dependent and -independent apoptotic cell death. Future study will be focused on the exploration of sensitivity to CHML between cells with functional p53 and cells with dysfunctional p53 status. Activation of p53-independent pathways requires

further investigation as well.

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