

The Effect of PM 701 on Mice Leukemic Cells: I - Tissue Culture Study of L1210 (*In Vitro*) II - *In Vivo* Study on Mice

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Abstract. Apoptosis was originally reported in 1972 and was described as programmed cell death. This process is very common in normal tissues with high proliferate activity such as bone marrow, and has also been implicated in the progression of a number of diseases, including AIDS, cancer and autoimmune pathologies. This study was conducted in two phases. The first phase used tissue culture of leukemia cells from mice in studying the effectiveness of agent (PM 701) on the behavior of these types of cells. This new agent, PM 701, proved to induce apoptosis of the cancer cells without affecting normal cells; the results showed that all tested concentrations of (PM 701) inhibited the growth of the cancer cells (L1210), with maximum effect at medium concentration. This finding is similar to our previous result in the use of (PM 701) as selective killing of tissue cultured lung cancer cells (A549). The second phase of this study was *in vivo* testing of the effect of (PM 701) in treating MFI mice that were infected with L1210 cells; the result of *in vivo* testing was satisfactory as *in vitro* effect at the tissue culture level.

Keywords: Leukemia cells, Tissue culture, Anticancer agent, Fibroblasts.

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Introduction

Although apoptosis is a goal in cancer therapy, apoptosis avoidance is hallmark. However, most common cancers do not easily undergo apoptosis and therefore are resistant to chemotherapy. Certain leukemias, Wilms, testicular cancer, teratocarcinomas and choriocarcinomas are curable^[1-4]. All these malignancies are apoptosis prone. In response to chemotherapeutic agents (e.g., DNA-damaging drugs) apoptosis-prone malignancies undergo apoptosis, a fast and active form of cell death. Once relapsed, leukemia, for example, may become apoptosis reluctant and therefore refractory to further chemotherapy^[5]. Similarly, although some testicular cancer is apoptosis prone and curable in most cases, some patients develop progressive disease despite treatment because of a defect in apoptotic pathway^[4,6]. Correlation between apoptosis and therapeutic response indicate that apoptosis, indeed, is a goal of cancer therapy^[7-10].

It is of primary importance to find an anticancer agent that kills cancer cells without unacceptable toxicity to the patient's own tissues and/or functions. Although induction of apoptosis determines responses to cancer therapy, this approach is limited by lack of selectivity in the available apoptosis-inducing agents. Furthermore, most cancers, almost by definition, are resistant to apoptosis, growth arrest and cell senescence. Therefore, the rational of drug combination (at mechanism-based doses and sequences) aimed at matching targets of selective cancer cell killing (apoptosis) with normal cells preservation. All new therapies aim to ensure this selectivity, by combining apoptosis and senescence-inducing agents with the inhibitors of apoptosis while at the same time protecting normal cells as tissue-selective therapy^[11].

The best approach to evaluate the effect of a new material is *in vitro* by utilizing the growing mammalian cells at tissue culture level and not on the living organism^[12-16]. This was applied in our laboratory at the Tissue Culture Unit (TCU), King Fahd Medical Research Center (KFMRC). Where we previously tested at tissue culture level the effect of (PM 701) as an anticancer agent on human lung cancer cells^[17] and in the present study on mice leukemia cells L1210 and documented the selective anticancer effect of PM 701, in causing programmed cancer cell death (apoptosis) with anti-apoptosis effect to normal tissues^[17]. The first aim of this research was to study at tissue culture level the effect of our novel agent (PM 701) on another type of cancer cell L1210, and secondly to study the use (PM 701) as a selective anticancer agent^[17]. The second aim is to present our findings of the *in vivo* study of this agent on MFI mice infected with L1210 cells.

Materials and Methods

Media

The following commercially available media were prepared according to published literature, these include:

RPMI (1640) (10% FCS) - RPMI is a multipurpose medium that was used for cultivation of mammalian cells^[18].

Phosphate Buffer Saline (PBS) is a phosphate-buffered physiological saline solution that is calcium- and magnesium-free^[16-18].

Trypsin^[16-18].

Examined Media

The examined agent is a natural substance, readily available, inexpensive, sterile, and non-toxic when subjected to chemical and microbiological testing^[17]. This agent was named (PM 701)^[17] and added to the ordinary media with medial concentrations ranging from 1:100 to 1:10,000.

1 ml substrate: 100 ml media, which is called - 2 PM 701

1 ml substrate: 1,000 ml media, which is called - 3 PM 701

1 ml substrate: 10,000 ml media, which is called - 4 PM 701

Cell Lines

Mice Leukemia Cells L1210 - Mice leukemia cells (L1210) were obtained by cell strain from American Type Culture Collection (ATCC) (Rockville, Maryland), available in the cell bank of the TCU, KFMRC, King Abdulaziz University (KAU), Jeddah.

In Vitro Proliferation of L1210 Cells Incubated in PM 701

1. L1210 cells were prepared *in vitro* in RPMI (1640) supplemented with 10% heat-inactivated fetal calf serum.
2. The cells were dispensed in 24 wells plate, 1×10^5 /ml in each well.
3. Cells were incubated 24 h in normal media, then the cells were divided to two groups:
 - I - Cells inoculated by PM 701 with different concentration (10^{-2} , 10^{-3} , 10^{-4}) and
 - II - Cells were growing in normal control medium for comparison.
4. Cells were collected by trypsinization.

5. *In vitro* proliferation assays were performed to compare the growth rate of the two groups of cells. The numbers of viable cells were counted using Hemacytometer and Trypan blue staining (0.4%)^[18].
6. For fixing and staining of cells each group of cells were plated onto Petri dishes in RPMI (1640) for 24 h, then the media changed with examined media (with different concentrations) and control media, then incubated at 37...C for 24 or 48 or 72 or 96 or 120 h.
7. Each group of cells was fixed in 4% formaldehyde for 5 min at room temperature after double washing with 1 × PBS each for 5 min.
8. Cells were then stained with Coomassie blue for 5-10 min followed by repeated washing with tap water^[16, 17].
9. For live experiment: Leukemia cells were allowed to grow in RPMI (1640) media for 24 h. Cells were imaged as a control for 5-10 min at controlled conditions for live experiment, the normal media was then changed with examined media during time-lapse images of living cells^[16, 17], for 90 min.

Live images were typically recorded using a CCD camera and saved in PC computer. Images were processed using computer programs. The data had been analyzed by using statistics computer programs.

***In Vivo* Study the Effect of PM 701 on Tumor Model**

Male MFI mice with approximately 25-30 g weight were purchased from the Animal House in KFMRC, KAU in Jeddah. Animals were housed in standard cages at the Animal House and allowed to acclimate to their surroundings for 7 days prior to the experiment. The mice had free access to drinking water and food during the experiment. All animals received humane care according to ethical requirements approved by the *Animals Research Ethic Committee of KAU*.

Lymphocyte leukemia, mouse cell lines L1210 were maintained in RPMI (1640) culture medium (10% FCS) before being inoculated into MFI mice.

To address the influence of the PM 701 on *in vivo* tumor development, we compared the tumorigenicity of L1210 cells in MFI mice.

Animals were divided to four groups:

- 1) Experimental group that were injected intraperitoneal with a dose of 0.2 ml of leukemia cells (1×10^5 /ml of media) and treated with orally administration of (PM 701) (15% in DW) after four days of infection.
- 2) Control animals received PM 701 only (15% in DW).
- 3) Control mice were injected with L1210 cells (same as pervious dose) without treatment.
- 4) Control mice drank only distilled water.

Animals were sacrificed 1- to 6-weeks following infection. Post-experimental tumor appearance and other physical observations as well as the mouse survival were reported, complete with color slide photo-documentation of animals at alternative periods.

Four types of specimens were collected from each group of animals each week before killing: Blood smear, blood serum, interprotineal fluid smear, and specimens for tissue culture investigations. Light and electron microcopies histopathology specimens included: Liver, lung, kidney, spleen and some tumors (these results will be published in paper II). Mice were anaesthetized with 4 ml ketamine & Section (3:1). When the animals were fully anaesthetized, they were sacrificed after blood had been collected from the jugular vein.

Results

I - In Vitro Proliferation Assays

For this task, the effect of PM 701 was examined on mice's leukemia cells (L1210). The results were compared with control (non-treated leukemia cells), where the cancer cells were incubated in ordinary media.

The *in vitro* growth behavior of L1210 cells appears to have similar initial fast growing phase (the first 24 h) in the two groups, whereas no difference in proliferation occurred between the experimental and control group in the initial phase. In contrast, L1210 incubated in PM 701 for more than 24 h exhibited a conspicuous 50% slower fast-growing step than the control (Fig. 1 and 2).

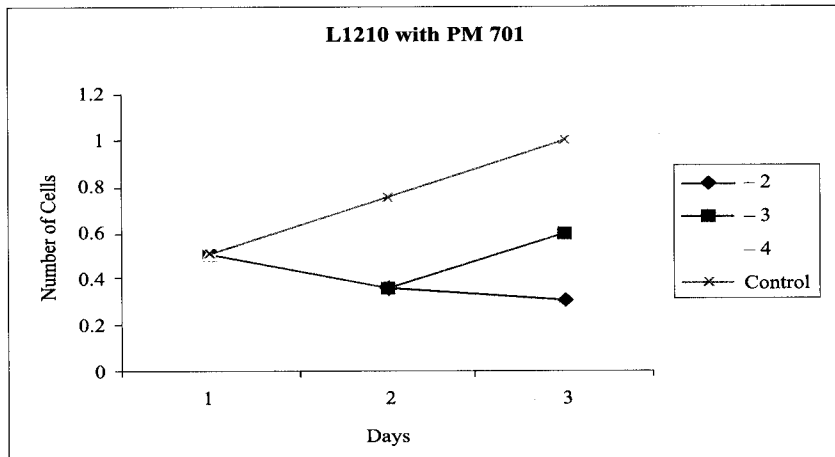


Fig. 1. Curves show the drop in the growth rate of L1210 cells when incubated in PM 701 compared with L1210 when incubated in ordinary medium.

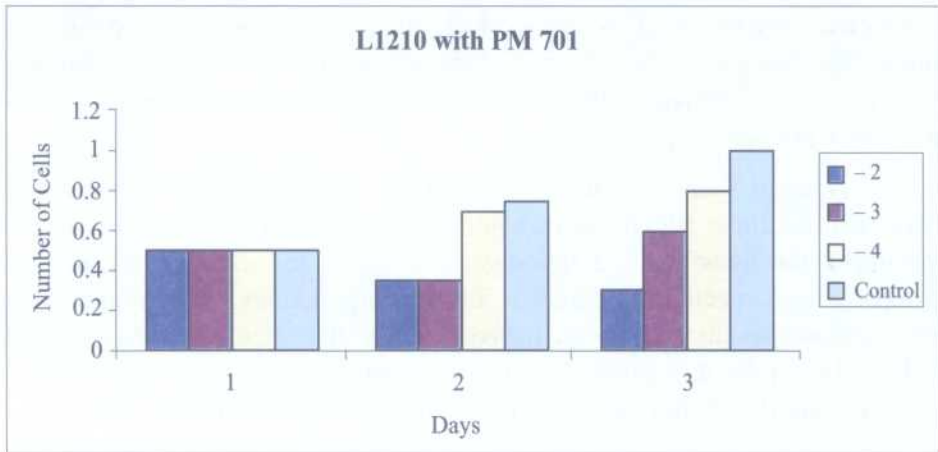


Fig. 2. Diagram indicates the proliferation differences of L1210 cells incubated in different concentration of PM 701 compared with control L1210 incubated in ordinary medium.

The new media that contained the examined substrate attacked the leukemia cells when incubated in it. This was proven by the next experiments that were given different parameters for the reaction of cells to the examined media.

Fixed and Stained Cells

This experiment shows the degree of injury and discomfiture of the leukemia cells from the incubated PM 701 medium; the effect on the cells includes the growth and reaction of cells and the size and number of cells for incubation longer than 24 h.

The images of the fixed and stained cancer cells (L1210) showed that the cell shrinkage occurred when incubated in media containing the examined substrate with various concentrations for 24 h compared with control media (Fig. 3-5), also the cancer cells became very rare when grown in PM 701 for a long period (72 h) result not shown. The images of fixed cells showed blebbing, which indicates that the examined substrate attacks the cancer cells causing cell apoptosis (Fig. 6).

Live Experiment

This experiment showed the direct effect of PM 701 medium on living cells. Cancer cells were allowed to grow in ordinary media for 24 h, cells were imaged in this media as a control for 15 min (Fig. 7a & b), then the media was changed with the examined media under live conditions for cells imaging. This live experiment showed the decrease in the size of cells incubated in PM 701 (Fig. 7c-f). The cancer cells incubated in PM 701 showed that the substrate

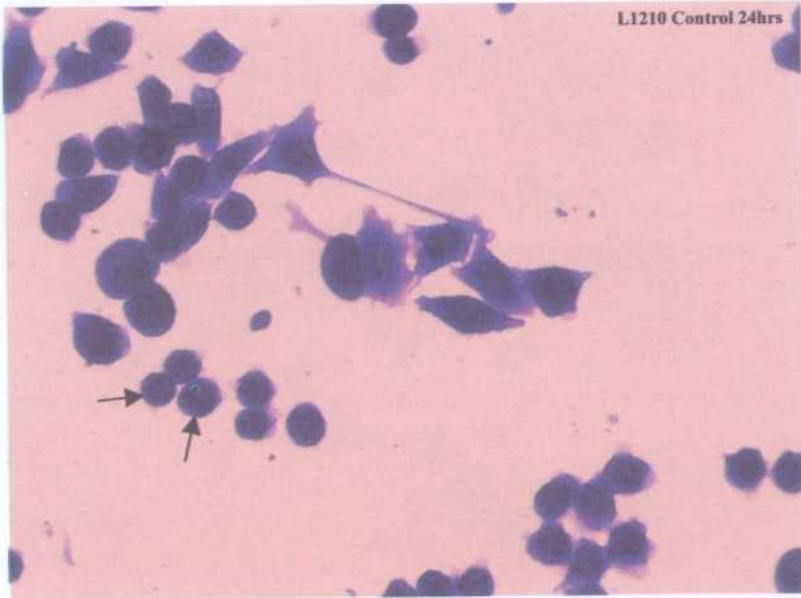


Fig. 3. L1210 cell incubated in ordinary medium as a control for 24 h \times 40, scale par 500 μ m.

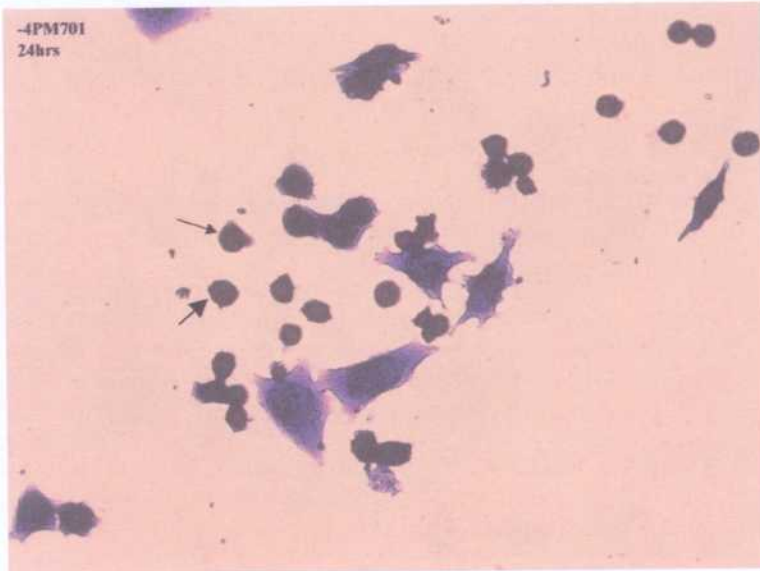


Fig. 4. L1210 incubated in -4 PM701 for 24 h, cells showed shrinkage and started to degenerate (arrows) \times 40, scale par 500 μ m.

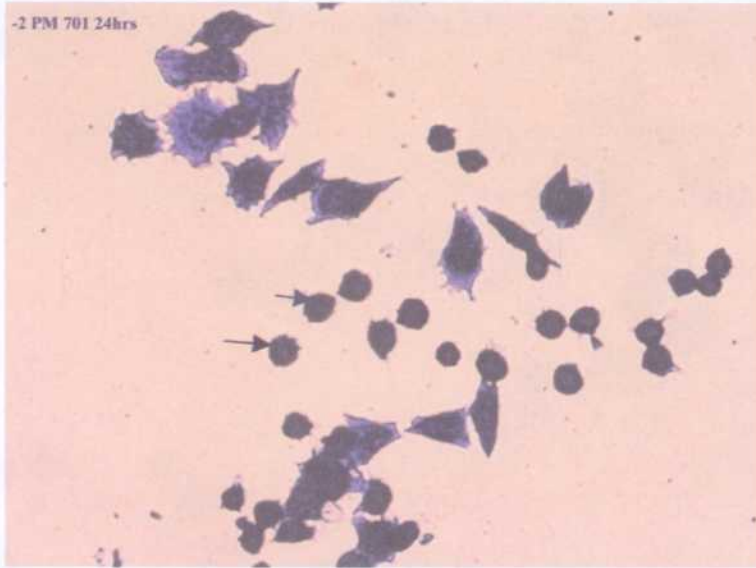


Fig. 5. L1210 incubated in -2 PM701 for 24 h, cells showed shrinkage and degenerated (arrows) $\times 40$, scale par 500 μ m.

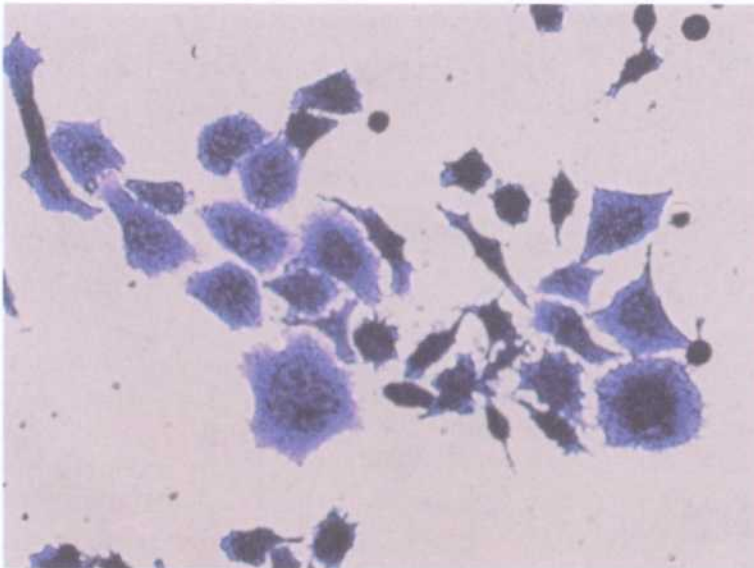


Fig. 6. L1210 incubated in -2PM701 for 24 h, the blebbing around cells indicate apoptosis (arrows) $\times 40$, scale par 500 μ m.

attacked the cell's nuclei, which was indicated by the appearance of condense chromatin after 30 min of incubation. This leads to the degeneration of cells (Fig. 7f), which could not be avoided by re-growing the cells in ordinary media. The severe effect on the nuclei of cancer cells limits the ability of cells to divide and survive, which signifies high efficiency in killing cancer cells.

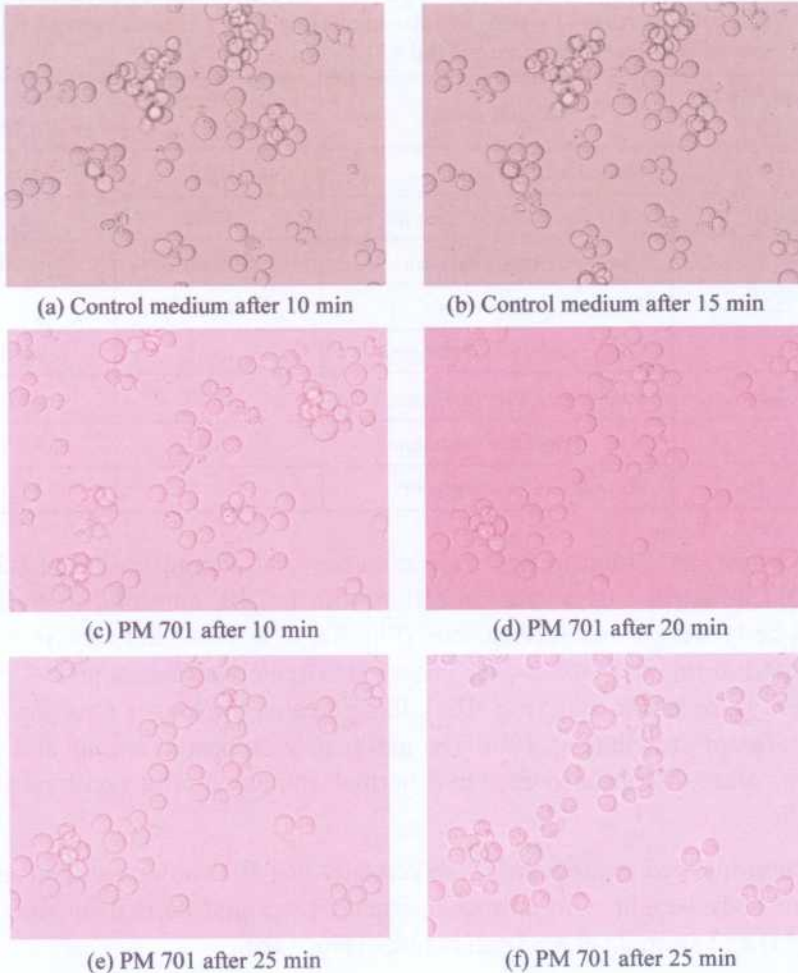


Fig. 7. The direct effect of PM 701 medium on living cells, cells appear to be discomforted after changing the medium, after 30 min the dense area inside the cells became visible which indicates the chromatin condensation $\times 40$, scale par $500\mu\text{m}$.

II - In Vivo Study of the Effect of PM 701 on MFI Mice

The effect of PM 701 was studied on MFI mice that were infected with L1210 cells. Animals injected with L1210 with no treatment exhibited a

remarkable increase in body weight (Table 1) with copious ascetic fluid (Fig. 8a & c). At autopsy, the animals in this group showed thin bodied muscular walls (Fig. 9a), and a huge white friable retroperitoneal mass (Fig. 9b). The gall bladder appeared abnormal and dark in color.

Table 1. The average difference in body weight between experimental and control mice, the dramatic increase in weight of mice inculcated with L1210 in spite of wasting of muscle wall due to huge ascetic fluid.

Control PM 701 (g)	Animal group	Control L1210 (g)	Exp. L1210 + PM 701 (g)
25.0	Started weights	30.00	30.0
30.0	After one week of infection	35.00	35.0
35.0	One week after treatment	40.00	37.0
35.1	Two weeks after treatment	40.20	38.0
35.3	Three weeks after treatment	40.30	40.0
35.5	Four weeks after treatment	43.30	42.0
37.1	Five weeks after treatment	45.25	42.5
40.7	Six weeks after treatment	—	45.0

In contrast, the group treated by continuous oral administration of (-2 to -3 PM 701) initiated 7 days after infection with L1210, appeared to have both normal body weight and survival rate (Fig. 8b, d, & f). At autopsy, only slight congestion of internal viscera was observed. The pelvic masses after 7 days of treatment were less in size (Fig. 10a), those treated for longer time showed no signs of tumor growth (Fig. 10b). The gall bladder appeared normal and yellow in color, other viscera also appeared normal compared with non-treated mice (Fig. 10c).

The non-injected group which received only PM 701 showed normal survival rate and body weight with normal internal viscera and normal muscular wall (Fig. 11a) and normal color of gall bladder (Fig. 11b).

It is of interest to mention that the lifespan of infected animals increased when treated with PM 701 compared with non-treated animals (Fig. 12).

Discussion

The balance between cell proliferation, cell differentiation and cell death determines the cell number in a population, as well as the size and stage of a tumor in the case of neoplastic masses.

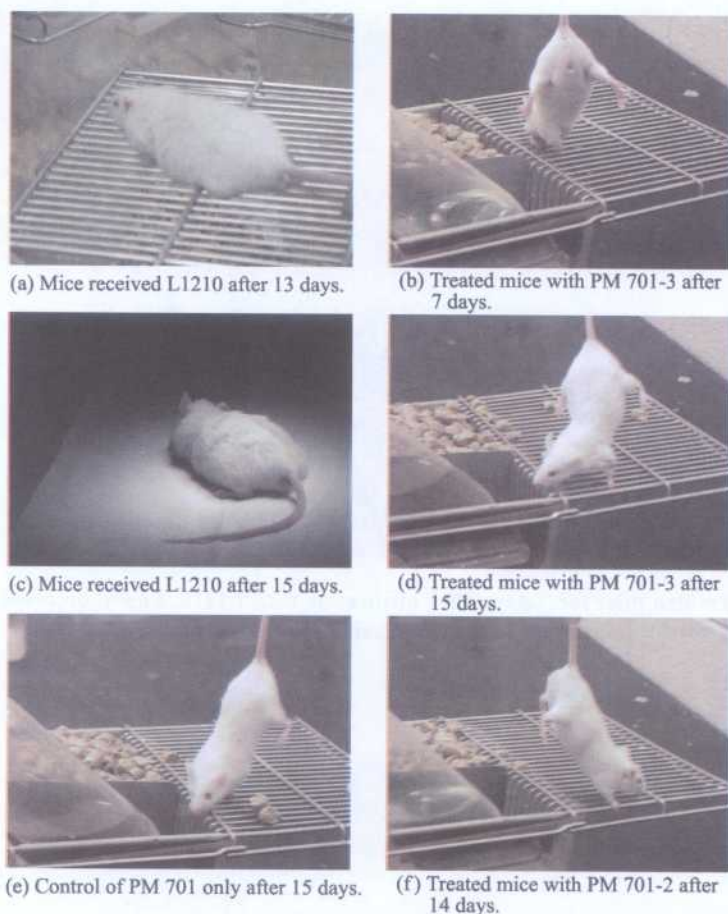


Fig. 8. The color photos show the differences in growth rate and animal survivals between the different groups of animals, note the unhealthy appearance of fur and huge ascetics in inoculated animals (a & c).



Fig. 9. Non-treated mice inoculated with L1210, a: Showed thin muscular wall (black arrow). b: Showed large friable lobulated white mass in pelvic and gastric regions (white arrows). The animal suffered from ascetics before opening.



Fig. 10(a). Treated mice for 7 days after inculcation with L1210 showed decrease in tumor growth compared to non-treated animals.

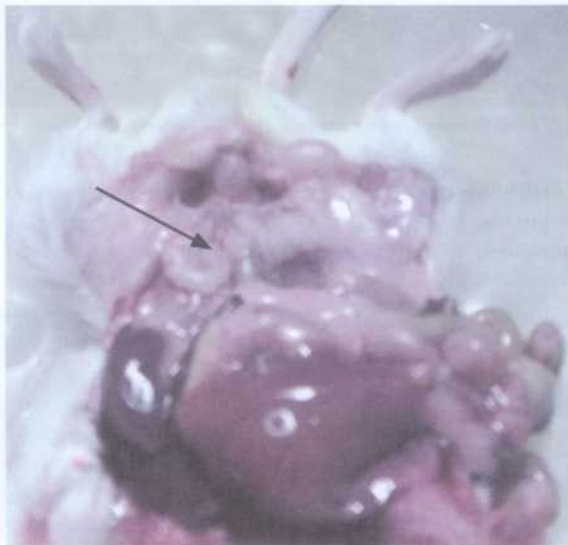


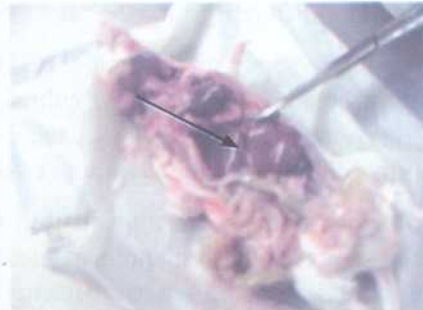
Fig. 10(b). Treated mice for 21 days after inculcation with L1210 showed normal pelvic region.



Fig. 10(c). Non-treated mice inoculated with L1210, after 3 weeks showed thin muscular wall with a huge ascetic fluid and abnormal viscera.



(a)



(b)

Fig. 11. Mice received PM 701 without inoculation: a) Normal muscular body wall and internal viscera; b) Normal gall bladder color (arrow).

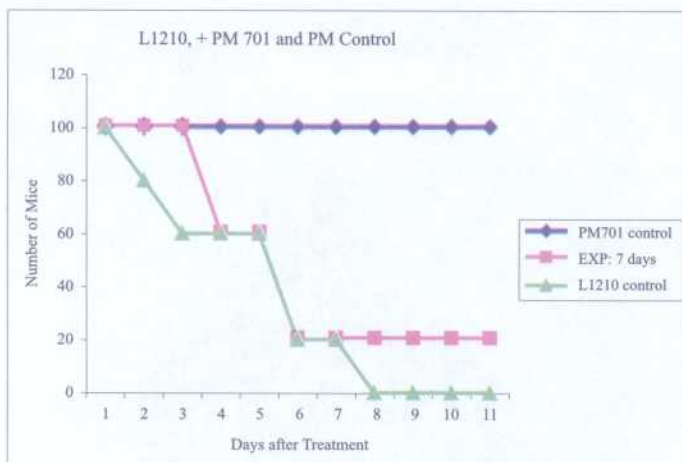


Fig. 12. The curve shows that the lifespan of inculcated animals increased when they were treated with PM 701 compared with non-inculcated animals that received only PM 701.

Therefore to increase our understanding of the pathogenesis of neoplasm, it is essential to investigate the regulation of cell proliferation and cell death.

Like proliferation, apoptosis plays a major role in the cell turnover of normal and neoplastic tissue^[19-21].

Apoptosis is a programmed cell death characterized by a variety of changes including; loss of cellular membrane phospholipids symmetry, chromatin condensation, and mitochondrial swelling and which eventually leads to damage and fragmentation of DNA. This process resulting in cell death is distinctly different from necrosis^[20,22].

In the previous study, we reported the effect of the substance (PM 701) on lung cancer cells *in vitro* at tissue culture level. Our findings demonstrated that (PM 701) could selectively kill the cancer cells (apoptosis), sparing and flourishing the normal fibroblast with maximum effect at medium concentration^[17].

In this study, we are presenting the further clear evidence of the apoptosis effect of (PM 701) on another type of cancer cell, mice leukemia cells (L1210). (PM 701) was examined at tissue culture level on cancer cells, L1210. The results were compared with control non-treated leukemia cells incubated in ordinary media.

This apoptotic effect of (PM 701) on cancer cells is clearly evidenced by the morphological changes seen in the experimental cells, including cell shrinkage, chromatin condensation, and blebbing. The general effect of (PM 701) on

neoplastic cells seems to be irreversible and it is impossible to recover them even when re-incubated in ordinary media due to severe damage of the nuclei limiting the ability of cells to divide and survive.

These results correspond with the definition of apoptosis given by Kerr *et al.*, 1972,^[20] when he said that apoptosis is programmed cell death characterized by cellular changes, including cell shrinkage, membrane blebbing, and chromatin condensation. In the nuclei of apoptotic cells DNA also is cleaved into oligonucleosomal-sized fragments.

The mechanism of apoptosis induction of (PM 701) is not clear but apoptosis can be induced by various intracellular signals, including growth factor deprivation^[22] and activation of cytokine receptors^[23,24]. Several compounds have been reported to cause apoptosis by enhancing or suppressing these signals^[25-28]. Evaluations of these require future studies on this substrate.

Furthermore, the *in vivo* study on the animal models indicated that (PM 701) has the ability to limit cancer progression in treated animals, which means that it has a favorable antimitotic effect.

Hence, it is apparent from the results of these experiments that the substrate (PM 701) is an effective and selective anticancer agent evidenced by its ability to induce apoptosis in neoplastic cells while sparing normal cells. In addition (PM 701) is an inexpensive, natural, readily available, sterile, non-toxic substance.

The success we have achieved in the use of this agent as anticancer with the safe and favorable effects on normal tissue *in vitro* and *in vivo*, makes this substrate a promising agent in the treatment of human cancer.

Conclusion

The role of PM 701 in cancer cell apoptosis has been supported by confirmed evidence from the present study in cell culture and animal model. The study also confirmed the tissue culture results of the previous experiments performed at TCU in KFMRC at KAU, Jeddah^[17].

Our study confirmed not only the *in vivo* effect of PM 701 as anticancer agent, but also the therapeutic effect of PM 701 as anticancer in metastasis models by histopathology study, confirmation is still in progress.

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