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***In vitro* Anticancer Agent**
I-Tissue Culture Study of Human Lung Cancer Cells A549
II-Tissue Culture Study of Mice Leukemia Cells L1210

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Abstract: Chemotherapy and radiotherapy are still the main weapons in the scientist's war against malignant disease. However, the results are far from the ideal treatment, which can be defined as 'the treatment that can selectively kill the malignant cell sparing the normal healthy tissue and the function of the vital organs'. Tissue culture of human lung cancer cells and mice leukemia cells were compared with that of normal (human skin fibroblasts) in studying the effect of PM 701. This agent proved to induce apoptosis of the cancer cells without detrimental effect on normal cells; through its effect on the nuclei, limiting the division of cells, causing degeneration in apoptotic manner. PM 701 exhibited nourishing effects on normal skin fibroblasts. This implies that this agent may have selectively cancer-cell killing- and reparative-effect on normal dividing cells. The present study represents the first experience in using PM 701 as a selective anti-cancer agent at tissue culture level. PM 701 is a natural product, readily available, cheap, sterile and non-toxic according to chemical and microbiological testing and proved effectiveness of this agent is reproducible on both cell lines used in the experiments.

Key words: Lung cancer, leukemia cells, anticancer, fibroblasts

Introduction

Lung cancer is considered to be an aggressive disease that affects a large number of patients yearly. It is considered to be one of the leading causes of cancer related deaths worldwide. It kills roughly thousands of patients every year. The global incidence of lung cancer is 1, 240 and 00 (i.e., 901,746 new cases in men and 337,115 cases in women worldwide in 2001), in 2003 the incidence was reported to be 170,000 cases per year in United States (Smith and Khuri, 2004; Pisick *et al.*, 2003; Marx, 2004; Schottenfield, 1996; Jemal *et al.*, 2002, 2003; Steward and Kleiheus, 2003).

Although apoptosis is a goal in cancer therapy, apoptosis avoidance is a hallmark. However, most common cancers do not easily undergo apoptosis; therefore, are resistant to chemotherapy. Certain leukemia, Wilm's tumor, testicular cancer, teratocarcinomas and choriocarcinomas are curable (Frei, 1985; Cohn and Herzog, 2000; Jones and Vasey, 2003; Mayer *et al.*, 2003). All these malignancies are apoptosis prone. In response to chemotherapeutic agents (e.g., DNA-damaging drugs) apoptosis-prone malignancies undergo apoptosis, fast and active form of cell death (Prokop *et al.*, 2000).

Correlation between apoptosis and therapeutic response indicate that apoptosis, indeed, is a goal of cancer therapy (Martin and Green, 1994; Houghton, 1999; Sellers and Fisher, 1999; Spierings *et al.*, 2003).

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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 aimed to ensure this selectivity, by combining an apoptosis and senescence-inducing agents with the inhibitors of apoptosis at the same time protect normal cells as tissue-selective therapy (Blagosklonny, 2004).

The best approach to evaluate the effect of new materials should be *in vitro* by utilizing the growing mammalian cells at tissue culture level and not on the living organism (Giaever and Keese, 1986; Alberts *et al.*, 1989; Cooper, 1997; Khorshid, 2001). This was applied in our laboratory at the Tissue Culture Unit (TCU), King Fahd Medical Research Center (KFMRC). We tested PM 701 as an anticancer agent on cultured lung cancer cells (A549); on mice leukemia cells (L1210); and at the same time, we tested its effect on the cultured normal skin fibroblast.

The study presented the first experience in using this alternative agent at the tissue culture level. Confirmed with documented evidence, the selective anticancer effect of PM 701 caused programmed cancer cell deaths (apoptosis) with anti-apoptosis effect to normal tissues. The aim of this research was to study at tissue culture level the effect of our novel agent (PM 701) on different types of cancer cell as a selective anticancer agent.

Materials and Methods

Media

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

- Ordinary media, (MEM) (10% FCS): Minimal essential medium (MEM) is a rich, multipurpose medium that was used for cultivation of human lung cancer cells (A549) and human normal fibroblasts (Pollared and Walker, 1989; Khorshid, 2005; Khorshid *et al.*, 2005).
- RPMI (1640) (10% FCS): RPMI (1640) was used for cultivation of mice leukemia cells L1210 (Pollard and Walker, 1989; Mushref *et al.*, 2005).
- Phosphate-buffered saline (PBS) is a phosphate-buffered physiological saline solution that is calcium- and magnesium-free solution (Cooper, 1997; Khorshid, 2001; Pollared and Walker, 1989).
- Trypsin: (Cooper, 1997; Khorshid, 2001; Pollared and Walker, 1989).
- PM 701 is a natural product, easily available, cheap, sterile and non-toxic according to our chemical and microbiological testing.

Examined media

The examined agent, PM 701, was used by adding it to the each ordinary media with different concentrations ranging from 1:10 to 1:100,000:

| | |
|---|-----------|
| 1 mL PM 701: 10 mL media, which is called | -1 (high) |
| 1 mL PM 701: 100 mL media, which is called | -2 |
| 1 mL PM 701: 1,000 mL media, which is called | -3 (mid) |
| 1 mL PM 701: 10,000 mL media, which is called | -4 |
| 1 mL PM 701: 100,000 mL medium, which is called | -5 (low) |

Cell Lines

Human Lung Cancer Cells Line

Human lung cancer cells, non-small cell carcinoma (A549) was obtained from cell strain from American Type Cultural Collection (ATCC), available in the cell bank of TCU at KFMRC.

Mice Leukemia Cells L1210

Mouse leukemia cells (L1210) was obtained from cell strain from ATCC (Rockville, Maryland), available in the cell bank of TCU.

Human Skin Fibroblast Cells

The normal human specimens (human skin fibroblasts) were obtained from King Abdulaziz University Hospital (KAUH) after circumcision operations. The specimens were transported immediately within 5 min after excision in previously prepared bottles of MEM media.

In vitro Proliferation of Cells

- Human lung cancer cells (A549) and human skin fibroblasts cells were suspended in culture medium MEM where mice leukemia cells (L1210) cells were prepared in RPMI (1640) medium.
- The cells were dispensed in 24 wells plate, $1 \times 10^5 \text{ mL}^{-1}$ in each well.
- Each group of cells was incubated 24 h in suitable media. Afterward each group of cells were divided to two subgroups:
 - I - Cells inoculated by MP 701 with different concentration and
 - II - Cells were growing in normal control medium for comparison.
- Cells were collected by trypsinization.

In vitro proliferation assays were performed to compare the growth rate of all groups of cells. The numbers of viable cells were counted using Hemcytometer and Trypan blue staining (0.4%) (Khorshid *et al.*, 2005; Pollard and Walker, 1989). The data was analyzed using statistical computer programs.

Fixing and Staining of Cells

- Each group of cells was plated onto Petri dishes in suitable media for 24 h then the media changed with examined media (with different concentrations) and with the control media then cells incubated at 37°C for 24 or 48 or 72 or 96 or 120 h.
- Each group of cells fixed in 4% formaldehyde for 5 min at room temperature after double-washing with PBS 1 x each for 5 min.
- Cells stained with Coomassie blue for 5-10 min followed by repeated washing with tap water (Cooper, 1997; Khorshid, 2001; Khorshid *et al.*, 2005).

For Live Experiments

Cells were allowed to grow in suitable media for 24 h. Cells were imaged as a control for 5-10 min at controlled conditions meant for live experiments, the normal media then changed with examined media during time-lapse images of living cells (Khorshid, 2001; Khorshid *et al.*, 2005; Mushref *et al.*, 2005) for 1-1½ h.

Live images typically were recorded using a CCD camera and saved in personal computer (PC). Images were processed using computer programs.

Results

In Vitro Proliferation Assays

For this part of experiment, the effect of PM 701 was examined on human lung cancer cells (A549) and mice leukemia cells (L1210) cells. The results were compared with positive and negative

control. The substrate effect was examined on normal cells (human skin fibroblasts) and on cancer cells that were incubated in ordinary media.

Although the results showed that PM 701 destroyed the cancer cells when added to the incubated media. There is satisfactory evidence that the same substrate did not cause any harm to the normal cells but flourished it.

This was proved by the following experiments that were given different parameters for the reaction of cells to the media.

Fixed and Stained Cells

This experiment showed the degree of injury and discomfiture of the cells in the incubated PM 701 medium. Human lung cancer cells (A549) or mice leukemia cells (L1210) incubated in suitable media for 24 h were compared with the cancer cells incubated in different concentration of examined substrate. It was well noticed that the low concentration of PM 701 (-4 and -5) affected the growth of the cells (Fig. 1 and 2) as the high concentration (-1). The best effect appeared near the middle concentration (-2 and -3) (Fig. 3 and 4); whereas, the effect on the cells included the growth and reaction and the number of cells.

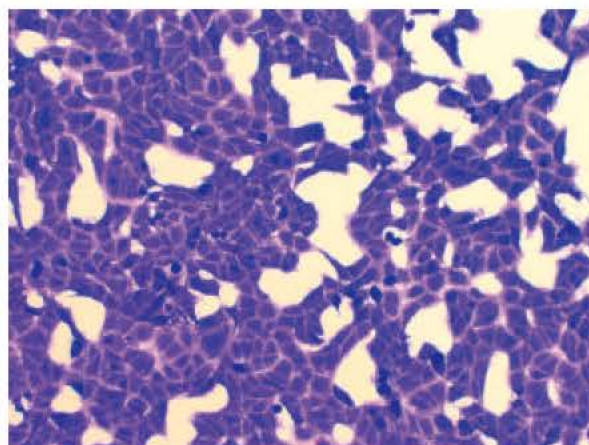


Fig. 1: A549 incubated in -4 PM 701 for 24 h \times 20. Scale bar 200 μ m

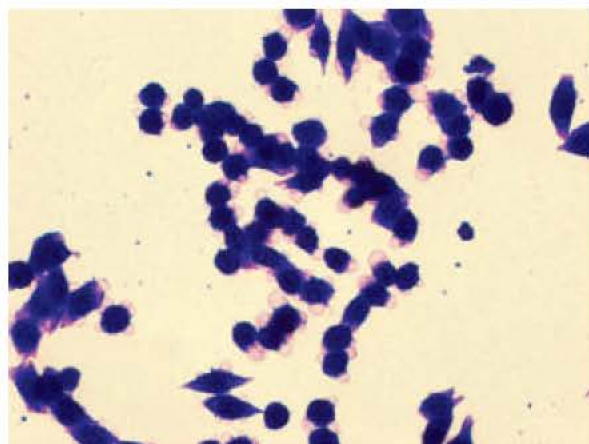


Fig. 2: L1210 incubated in -4 PM 701 for 24 h \times 40, the blebbing around cells indicate apoptosis. Scale bar 500 μ m

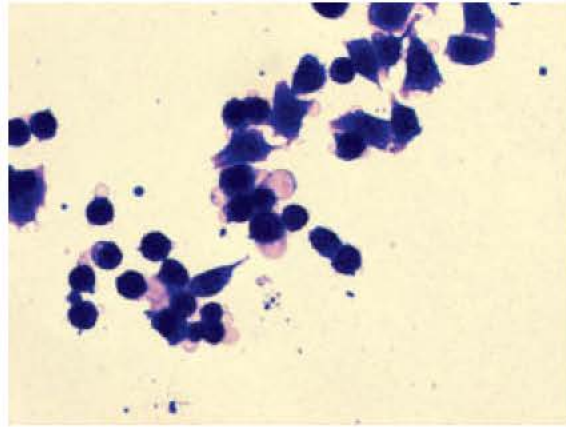


Fig. 3: L1210 incubated in -2 PM 701 for 24 h $\times 40$, the blebbing around cells indicate apoptosis. Scale bar 500 μm

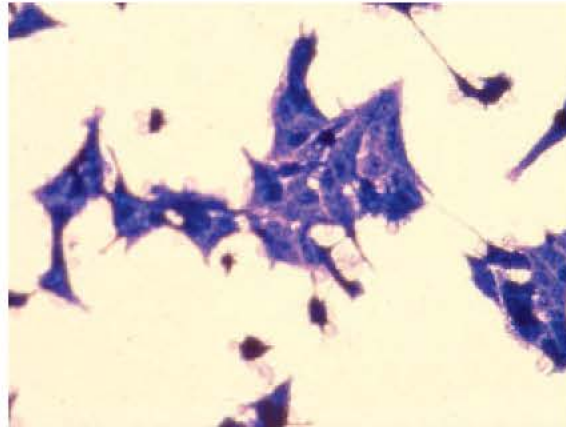


Fig. 4: A549 incubated in -3 PM 701 for 24 h $\times 20$, the blebbing around cells indicate apoptosis. Scale bar 200 μm

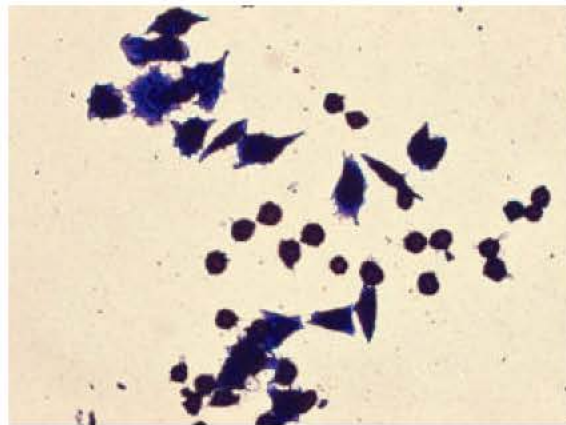


Fig. 5: L1210 incubated in -2 PM 701 for 24 h $\times 40$, cells appeared shrinkage and started to degenerate. Scale bar 500 μm

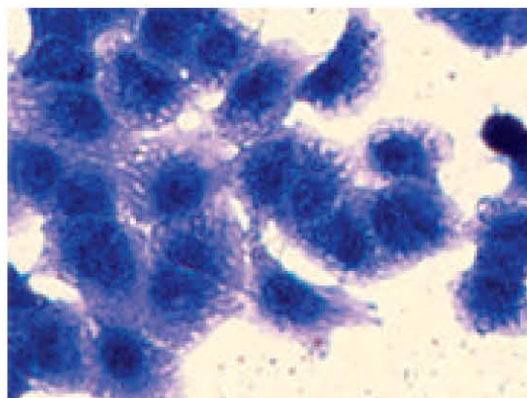


Fig. 6: A549 incubated in -3 PM 701 for 24 h \times 40, cells appeared shrinkage and started to degenerate. Scale bar 500 μ m



Fig. 7: L1210 incubated in RPMI 1640 for 24 h \times 40. Scale bar 500 μ m

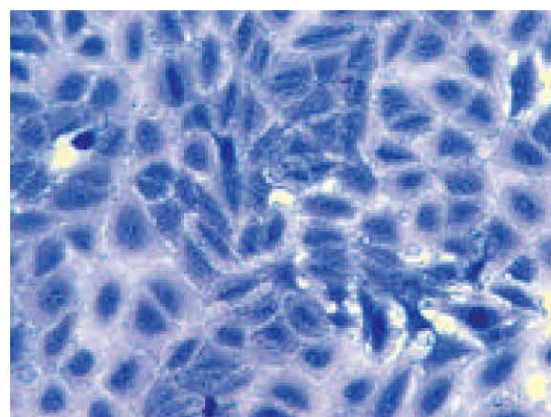


Fig. 8: A549 incubated in MEM for 24 h \times 20. It was well observed the high ability of cancer cells to divide. Scale bar 200 μ m

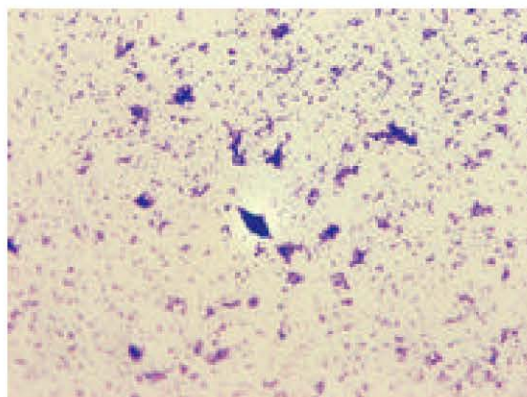


Fig. 9: A549 incubated in -3 PM 701 for 96 h \times 20, note the lost of cells from the field. Scale bar 200 μ m

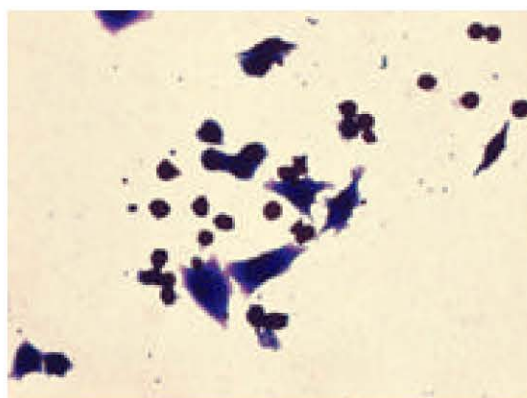


Fig. 10: L1210 incubated in -3 PM 701 for 24 h \times 40, note dense area inside the cells. Scale bar 500 μ m

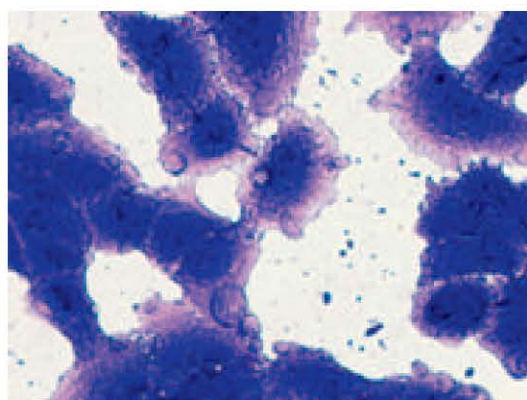


Fig. 11: A549 incubated in -3 PM 701 for 24 h \times 40, note that the cancer cells nuclei attacked by PM 701, indicating by dense area inside the cells. Scale bar 500 μ m

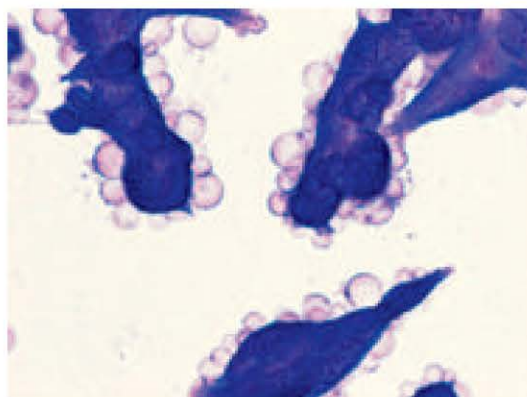


Fig. 12: A549 incubated in -3 PM 701 for 24 h \times 40, the blebbing around cells indicate apoptosis. Scale bar 500 μ m

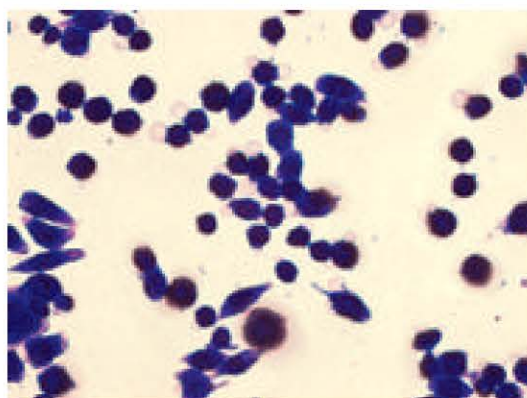


Fig. 13: L1210 incubated in -4 PM 701 for 24 h \times 40, the blebbing around cells indicate apoptosis. Scale bar 500 μ m

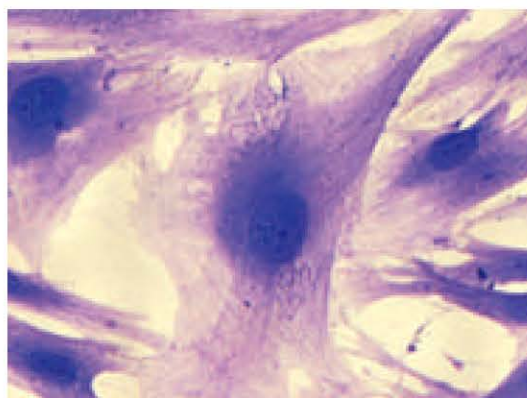


Fig. 14: Human Fore Skin cells incubated in (-2) PM 701 (24 h) -40 x, note the well spreading cells with obvious well developed cytoskeleton and organelles. Scale bar 500 μ m

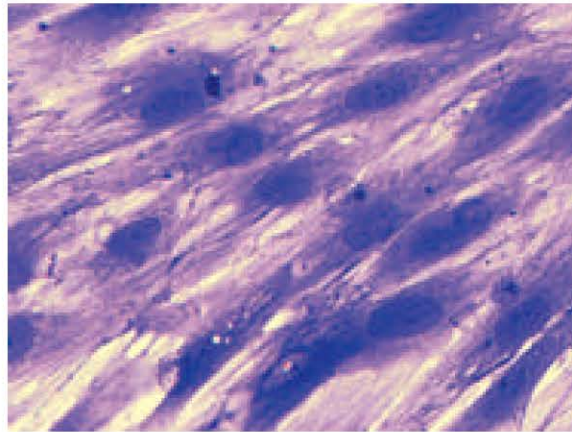


Fig. 15: Human fore skin cells incubated in (-) PM 701 (3 days) -40 x, note the adhesion between cells in the complete sheet. Scale bar 500 μ m

The fixed and stained cancer cells images showed cell shrinkage when incubated in media containing the examined substrate with different concentrations for 24 h (Fig. 5 and 6) compared with cells incubated in control media (Fig. 7 and 8). As well, the cancer cells became very rare when grown in PM 701 for long periods (96 h) (Fig. 9). Furthermore, this substrate damaged the nuclei of the cancer cells, which is indicated by chromatin condensation (Fig. 10 and 11).

The examined substrate attacked the cancer cells; cells appeared destroyed and decreased in number, have blebbing, which indicate the cell apoptosis (Fig. 12 and 13).

Whereas the normal cells (human skin fibroblasts), appeared healthy with advanced growth and spreading when grown in PM 701 (Fig. 14) even when incubated for a long time (Fig. 15).

Live Experiment

This experiment showed the direct effect of PM 701 medium on living cells. Cancer cells and normal fibroblasts were permitted to grow in ordinary media for 24 h. Cells imaged in this media as a control for 5-15 min (Fig. 16 (1a and 1b)), then the media changed with the examined media in live conditions for cells imaging. This live experiment showed that the severe lethal effects of PM 701 on cancer cells started immediately after 5-6 min since adding the examined substrate (Fig. 16 (2b)). The live observations of normal fibroblasts incubated in PM 701 as a control, illustrated that the fibroblast cell retracts after adding the new media immediately by pulling its pseudopodia, which might be explained by the introduction of the cell to this substrate. The well spreading of the cell body of the fibroblasts indicated the positive reaction between the cell and this new substrate (Fig. 16 (1a-4a)). The cancer cells incubated in PM 701 showed that the substrate attacks the cell's nuclei, which is indicated by the appearance of pale ring around the nucleus of the lung cancer cell (Fig. 16 (2b)) and by the appearance of condense chromatin in leukemia cancer cells after 30 min of incubation (Fig. 17d, 4). This leads to the degeneration of cells (Fig. 16 (3b- 4b); Fig. 17d, which could not be reversed to recover the cells by re-growing the cells in ordinary media again. The severe effect on the nuclei of cancer cells limit the ability of cells to divide and survive, which is seems of high efficiency in killing cancer cells.

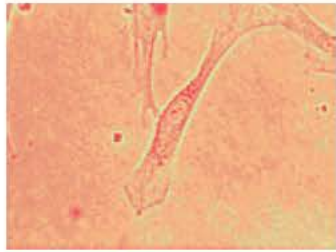


Fig. 16 (1a): Normal fibroblasts grow in MEM media for 24 h, then the cells imaged for 10 min in controlled conditions x 40, scale par 500 μ m

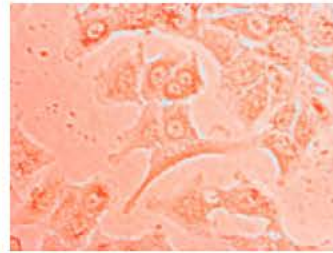


Fig. 16 (1b): Cancer cells grow in MEM media for 24 h, then the cells imaged for 10 min in controlled conditions x 40, scale par 500 μ m

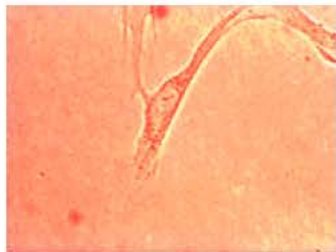


Fig. 16 (2a): Normal fibroblasts after changing the media by PM701. Note that the cell retracts x 40, scale par 500 μ m

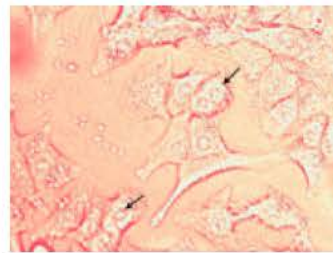


Fig. 16 (2b): Cancer cells after changing the media by PM701. The cancer cells nuclei attacked by PM701, indicated by the pale color surrounded the nuclei (arrows) x 40, scale par 500 μ m

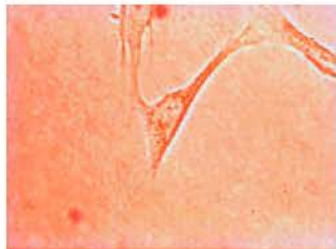


Fig. 16 (3a): Normal fibroblasts in PM 701, the cell body increased in size x 40, scale par 500 μ m

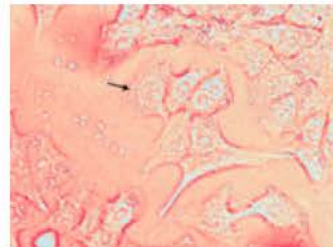


Fig. 16 (3b): Cancer cells in PM 701. Cells appeared to degenerated (arrow) x 40, scale par 500 μ m

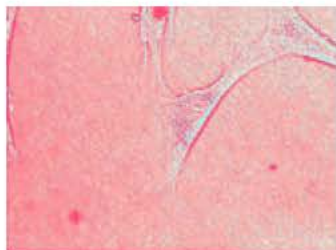


Fig. 16 (4a): Normal fibroblasts in PM 701, the cell body increased in size. x 40, scale par 500 μ m

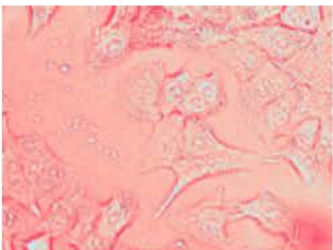


Fig. 16 (4b): Cancer cells in PM 701. Cells appeared to degenerated (arrow). x 40, scale par 500 μ m

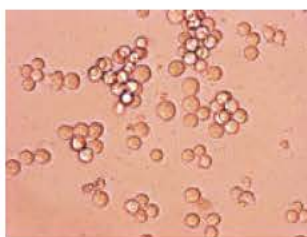


Fig. 17 (a): L1210 cells in RPMI1640 as a control (10 min). x 40, scale par 500 μ m

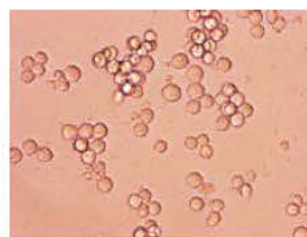


Fig. 17 (b): L1210 cells in RPMI1640 as a control (15 min). x 40, scale par 500 μ m



Fig. 17 (c): The direct effect of PM 701 medium on living L1210 cells, cells appear to be discomfiture after changing the medium (25 min), x 40, scale par 500 μ m

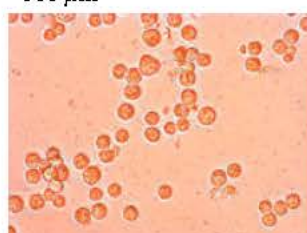


Fig. 17 (d): L1210 cells in PM 701 (30 min), the dense area inside the cells became visible which indicates the chromatin condensation x 40, scale par 500 μ m

Cells Count

The *in vitro* growth behavior of living cells and the viability of cells after incubated in PM 701 were studied using cells count method. Results show severe drop of cancer cells number when incubated in PM 701 compared with the number of control cells (cancer) that incubated in ordinary suitable media, (Fig. 18 and 19). Furthermore, the normal cells (fibroblasts) number shows normal progress, when incubated in PM 701 with very little depression in the first stages of incubation (Fig. 20). The depression of fibroblasts number incubated in PM 701 in the beginning of the experiment explained by the introducing of cells to the new media. While the fixed images indicate that normal cells (fibroblasts) comfort to the new media after short time and appeared healthy and spread more than in the ordinary media.

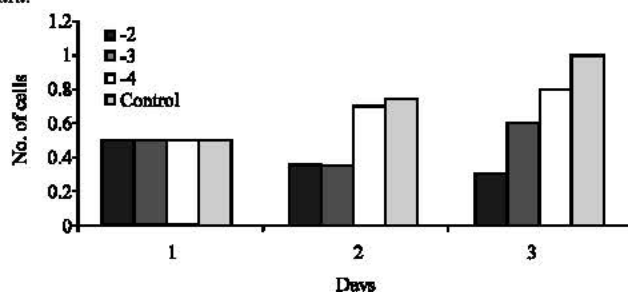


Fig. 18: Diagram indicates the proliferation differences of L1210 cells incubated in different concentration of PM 701 comparing with control L1210 incubated in ordinary medium

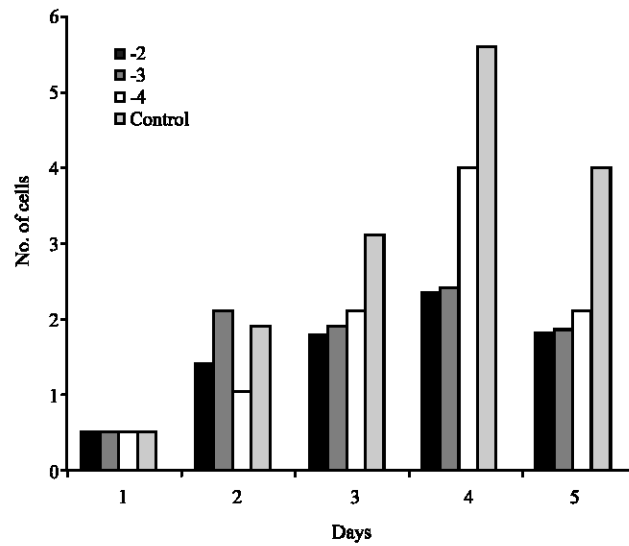


Fig. 19: The diagram show the same number of cancer cells incubated in PM 701 or MEM as a control for the first 24 h, whereas the control cells increase in number by increase the number of days, comparing with cells incubated in different concentration of PM 701, see day 4

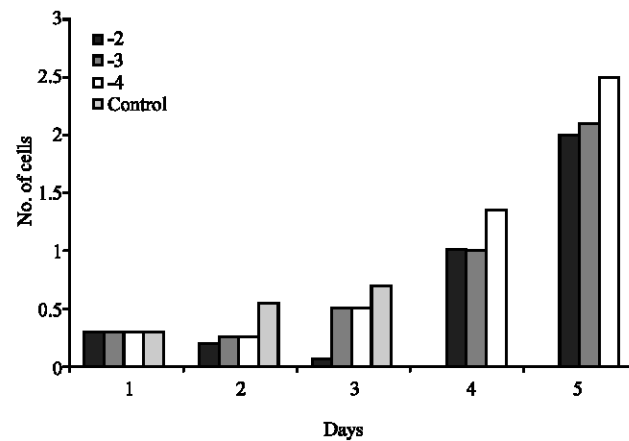


Fig. 20: The diagram indicates that there is no negative effect of different concentration (-2 or -3) of PM 701 on the cell number of fibroblasts comparing with fibroblasts incubating in MEM

Discussion

The achievement in the war against the malignant disease varies greatly, depending on the types of the neoplasm, the stage of the disease and the degree of histological favorability (Pisick *et al.*, 2003). 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.

Like proliferation, the apoptosis plays a major role in the cell turnover of normal and neoplastic tissue (Wyllie, 1980; Kerr *et al.*, 1972; Cummings *et al.*, 1997).

Apoptosis is programmed cell death characterized by variety of changes including loss of cellular membrane phospholipids symmetry, chromatin condensation, mitochondrial swelling and eventually

leads to damage and fragmentation of DNA. This process resulting in cell death is distinctly different from necrosis (Kerr *et al.*, 1972; Darzynkiewicz *et al.*, 1998).

In spite of the success in satisfactory control of some of the malignant disease and failure in the others with the available modalities, i.e., chemotherapy and radiotherapy (Pisick *et al.*, 2003; Blagosklonny, 2004), we remain far from the ideal treatment, which we can define it as 'the treatment that can selectively kills the malignant cell sparing the normal healthy tissue and the function of the vital organs'. Our study indicated that the PM 701 did fulfill the criteria of the ideal treatment for cancer cells *in vitro* as seen in killing lung cancer cells and mice leukemia cells while it has a flourishing effect on the normal fibroblast, with maximum effect at medium concentration.

Apoptosis can be induced by various intracellular signals, including growth factor deprivation (Araki *et al.*, 1990) and activation of cytokine receptors (Laster *et al.*, 1988; Nagata and Golstein, 1995). Several compounds have been reported to cause apoptosis by enhancing or suppressing these signals (Muthukkumar *et al.*, 1995; Yao and Cooper, 1995; Stefanis *et al.*, 1999; Fujino *et al.*, 2002; Suk *et al.*, 2003).

In the present study, the apoptotic effect of PM 701 on cancer cells is clearly evident by the morphological changes seen in our experimental works, which included cell shrinkage, chromatin condensation and blebbing with progressive decrease in the number of the cells in media till the disappearances of the cell in approximately 120 h, but the mechanism of apoptosis induction of PM 701 is not clear and may need further investigations. Where the general effect of PM 701 on neoplastic cells seems to be irreversible and it is impossible to recover it even when re-incubated in ordinary media due to severe damage of the nuclei limiting the ability of cells to re-divide and survive. This results correspond with the definition of Kerr *et al.* (1972), 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.

On the other hand, mixing PM 701 with normal fibroblasts media showed early precautionary reaction of the cells upon the contact with new substrate by withdrawal of their pseudopodia but with an increase in the size of cell body followed in the short time by normal reaction and flourishing (growth, spreading of cells and well developed cytoskeletons or other organelles) this flourishing effect was seen in the different concentration of the media (low and medium). While the high concentration of substrate was toxic to the cells, this toxicity can be understood as the cells in the culture media are isolated from body protective and regulatory mechanisms and immunity function of intact living organism so cannot tolerate the concentrated substrate, this is why different concentrations has to be used to test the effect of the substrate avoiding the full or high concentration.

These results were reproducible at the *in vitro* level may lead to successful alternative anticancer agent for human cancers other than radiotherapy or chemotherapy. Our study which is in progress at the *in vivo* level showed promising results of PM 701 as anticancer agent in cancer induced animal model, further confirmation is required to be published.

Conclusion

In this study, we obtained an anticancer substrate PM 701, which is natural, easily available, cheap, sterile, non-toxic and can cause selective cell death of cancer cells; it does not require high concentration as chemotherapy. PM 701 has flourishing effect on normal skin fibroblasts at the tissue culture media. The effect of PM 701 is reproducible on human lung cancer, A549 and on another cell line, mice leukemia cells, L1210.

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

Acknowledgments

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