CHAPTER- 7

IN VITRO CYTOTOXICITY OF THE COMPOUNDS ISOLATED FROM ENDOPHYTES WSEPF-3 AND DSEPA-1

INTRODUCTION

The valuable contributions of nature as a source of potential chemotherapeutic agents has recently been reviewed (Newman and Cragg, 2007). Cancer is the second major cause of deaths after cardiovascular diseases and there is a need to combat such diseases. It is commonly referred to disease in which normal cells undergo abnormal cell proliferation and differentiation. These abnormalities are due to mutations to cell genes, thus producing oncogenes and causing the tumor suppressor genes to lose its functions. Cancer has been considered a major cause for mortality worldwide. World Health Organization has reported that cancer has caused 7.4 million mortalities in 2004. This has been quite a concern as the death from cancer is projected to increase by estimation of 12 million deaths in year 2030 (WHO, 2009).

The search for natural products as potential anticancer agents dated since 1550 B.C. and surveying the period from 1981 to 2002. Newman et al., (2003) reported that >60% of the approved drugs for cancer treatments are natural products or derivatives of natural products. A large number of plants, marine and microbial sources have been tested as leads and many compounds have survived as the potential leads. Unfortunately there has been over exploitation of these plant species as sources of raw material for production of medicine leading to depletion of a number of plant species. It is necessary to reduce dependency on wild resources and find alternative sources for the production of these important biomolecules. Microbes associated with these plants (endophytes) may be the source of these important bioactive molecules and the way for conserving the important medicinal plants.
Other than plants, microorganisms also play a major role in anticancer drug discovery as natural products. Endophytes, which are microorganisms found in plants and can be described to have symbiosis interaction with its host (Tadychand White, 2009). One of the most important classes of microbial derived agents is the anthracyclines, with daunorubicin and its derivative doxorubicin (adriamycin) being the best known of these agents currently in clinical use; they are still major components of the treatment regimen for breast cancer (Arcamone, 2005) which was isolated from the Streptomyces species. Structures based on the epothilones, isolated from the extremely prolific Myxomycetales (Hofle and Reichenbach, 2005) are of great interest as potential antitumor agents due to their mechanism of action being the same as that of paclitaxel (vide infra). Paclitaxel and some of its derivatives represent the first major group of anticancer agents that is produced by endophytes a novel paclitaxel-producing endophytic fungus, _T. andreanae_, was discovered in _T. brevifolia_ (Strobel et al., 1993). Torreyanic acid, a selectively cytotoxic quinone dimer (anticancer agent), was isolated from a _P. microspora_ strain. This strain was originally obtained as an endophyte associated with the endangered tree _T. taxifolia_ (Florida torreya) as mentioned above (Lee et al., 1996).

Several population based studies showed that the people in South East Asian countries have much lower risk of colon, gastrointestinal, prostrate, breast and other cancers than their western counterparts (Dorai and Aggarwal, 2004) and it is thought that constituents of their diet may play a significant role in protection. Indeed, phenolic substances present in the fruits, vegetables, medicinal plants, have cancer chemo preventive activities, both in vitro as well as in vivo animal models (Surh et al., 1999; Mahmoud et al., 2000; Kim et al., 2004; Murakami et al., 2004).
Compounds that block or suppress the proliferation of tumor cells have the potential to function as anticancer agents. A quantitative method for measuring the cytotoxic action of carcinolytic agents on continuous human cell lines in a culture was developed by Eagle and Foley (1958). With the technical advances (Earle et al. 1950), the possible applications of monolayer cell culture have increased considerably. The development of a technique for the production of large quantities of replicate cultures from rapidly growing dividing cells and the establishment of an increasing number of cell lines, meant that the effects of various compounds could be studied directly on cell in vitro (Ambrose and Easty, 1967).

The beginning of modern era (1940-1950), cancer chemotherapy can be treated directly to the discovery of nitrogen mustard, a chemical warfare agent, as an effective treatment of cancer. Two pharmacologists, Louis Goodman and Alfred Gilman were recruited by the United States Department of Defense to investigate the potential therapeutic application of chemical warfare agents. Autopsy observations of people exposed to mustard gas had revealed the profound lymphoid and myeloid suppression. Goodman and Gilman (1984) reasoned that this agent could be used to treat lymphoma, a tumor of lymphoid cells. This was the first step to the realization that cancer could be treated by pharmacological agents (Goodman et al., 1984).

It is important to realize that the chemotherapeutic agents had been discovered essentially by cytotoxicity or by inhibiting the metabolic pathways crucial to cell division, but none was particularly specific to the cancer cell. Currently the search is continuing with the pharmaceutical industry to screen for new anticancer compounds from various sources; initially with the cytotoxic activity in vitro. Thus it plays a major role in the development of anticancer compounds.
Apoptosis

Apoptosis is the cell death process, which occurs during the development and aging of animals and also induced by cytotoxic lymphocytes (CTL). Apoptosis was initially characterized by morphological changes of dying cell. During apoptosis, cells shrink and the plasma membrane disappears, nucleus becomes condensed and fragmented. At the final stage of apoptosis the cells themselves are fragmented with all cellular contents. Mitochondria remain unchanged morphologically. This type of cell death is often hard to observe in vivo because the dying cells are rapidly phagocytized by tissue macrophages and this phagocytosis is clearly different from inflammation, when activated macrophages are recruited from outside the immediate area of death. The present chapter deals about the cytotoxicity activity of the active compounds isolated from endophytes on human liver cancer cell line (HepG-2) and chang liver normal cell line.

Materials and Methods

Reagents

1. MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide): 0.5 mg MTT/ml of serum-free DMEM medium.

2. Solubilizing solution: Dimethyl sulfoxide

3. Phosphate buffered saline (PBS) (pH 7.4): As described under cell culture reagents.

Test Compounds

Compound isolated from endophytic actinomycetes (Gallic acid) and endophytic fungi (C16H20O6) \((3R, 5S, 10aR)-3, 4a, 5, 6, 9, 10a\)-hexahydroxy -7methoxy -3-methyl-1H, 3H, 4H, 4aH, 5H, 10H, 10aH –naphtho [2, 3 -c] pyran-10-one were tested for cytotoxicity activities.
Drug preparation

The compound was dissolved in different concentrations (10 to 250 µg/ml) in 10% Dimethyl Sulfoxide (DMSO) to give a final concentration of DMSO not more than 0.5% and did not affect cell survival.

Cell viability test

The viability of cells was assessed by MTT assay (Mosmann, 1983) using HepG2 cell lines and chang liver cell lines.

Principle

The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able to take up the tetrazolium salt. The enzyme (succinate dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed and dissolved in DMSO solution. The colour developed is then determined in an ELISA reader at 570 nm.

Cytotoxicity Studies

The HepG-2 cells and chang liver cells were plated separately in 96 well plates at a concentration of 1 × 10^5 cells/well. After 24 h, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37 °C. After starvation, cells were treated with different concentrations of test compound (25-500µg/ml) and incubated for 24 h. At the end of the treatment period the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37 °C in a CO₂ incubator. The 50% inhibitory concentration value (IC₅₀) of the compound was identified for normal fibroblast cell line.
The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software.

**Results**

Cytotoxicity effect of the active compounds isolated from endophytes of *Withania somnifera* (WSEPF-3) and *Datura stramonium* (DSEPA-1) were studied using a chang liver cells and HepG-2 cancer cell line (Fig 7.1). The degree of toxicity of these test compounds towards the cell lines were determined using MTT assay. The assay is colorimetric based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan crystals.

The cytotoxicity activities expressed as percentage of cell viability in normal and HepG-2 cell line compared with cyclophosphamide and control. The cytotoxic assays showed a significant activity of the compound from endophytic fungi of *Fusarium cf solani* isolated from *Withania somnifera* on the viability of chang liver and HepG-2 cells *in vitro*, when compared to the cyclophosphamide and DMSO (control) treated cells. Different concentrations were tested varying between 10-250 µg/ml and it was determined that in the concentration of 250 µg/mL, the compound of the endophytic fungi was able to inhibit about 36.46 % of cell proliferation in HepG-2 cells and 77.97% of cells proliferation in normal Chang liver cells. In cyclophosphamide 10.16% (15µg/ml) of cells proliferated in HepG-2 and 12.55% in normal cells were observed (Fig 7.2& Fig 7.4).

The cytotoxicity effects of Gallic acid isolated from the extracts of endophytic actinomycetes were studied. The different concentrations (10-250 µg/ml) of compound showed
moderate cytotoxic activities against HepG-2 and normal Chang liver cell line. The results showed 56.37% (250 μg/ml) of cells proliferate in HepG-2 cell line and 89.50% (250 μg/ml) were observed in normal liver cells. The 11.28%, 12.83% of cells were proliferate in 15 μg/ml of cyclophosphamide in HepG-2 and normal Chang liver cells (Fig 7.3 & Fig 7.5).
Discussion

Metabolic products by endophytes could be influenced by the chemistry of their host plants (Huang et al., 2008; Mucciarelli et al., 2007; Aly et al., 2010). Several cytotoxic compounds have been isolated from endophytic fungi, showing the potential of these fungi in the search for antitumoral agents (Guimarães et al., 2008). During the long period of coevolution, some endophytes have the ability to produce similar or identical biological active compounds as their host plants, such as paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin and diosgenin (Zhao et al., 2011). Firáková et al., (2007) presented the overview of bioactive secondary metabolites with anticancer activity produced by endophytes, for example, camptothecin of a fungal endophytic isolate, podophyllotoxin, aryltetralignans of *Trametes hirsuta*, cytoskyrins of *Curvularia lunata* and macrolides of *Streptomyces* sp.

In the present study, the cytotoxic potential of the compounds ((3R,5S,10aR)-3,4a,5,6,9,10a-hexahydroxy-7-methoxy-3-methyl-1H,3H,4H,4aH,5H,10H,10aH-naphtho[2,3-c]pyran-10-one (C_{16}H_{20}O_{6}) and Gallic acid) isolated from endophytic fungi and endophytic actinomycetes extract of *Withania somnifera* and *Datura stramonium* respectively were assessed by MTT assay against Chang Liver Cells and HepG-2 cancer cell line. This assay is based on the reduction of yellow tetrazolium salt (MTT) by metabolically active cells to a dark blue formazan, and has been employed by many groups to measure the cytotoxic effect of any compound on cells. The results showed, compound from endophytic fungi (3R,5S,10aR)-3,4a,5,6,9,10a-hexahydroxy-7-methoxy-3-methyl-1H,3H,4H,4aH,5H,10H,10aH-naphtho [2,3-c] pyran-10-one (C_{16}H_{20}O_{6}) was significantly active against normal and HepG-2 liver cells. Meanwhile the Gallic acid isolated from endophytic *Streptomyces* species showed a moderate activity. Whereas Li et al., (2008c) investigated that endophytic actinomycetes associated with medicinally important plant reported forty one microorganisms from the *Streptomyces* displayed a significant
antitumor activity against HL-60 cells, A519 cells, BEL-7404 cells and P388D1 cells. Finally, other compounds with anticancer properties isolated from endophytic microbes were reported such as cytoskyrins, phomoxanthones A and B, photinides A-F, rubrofusarin B and (+) epiepoxydon (Brady et al., 2000; Isaka et al., 2001; Song et al., 2004; Klemke et al., 2004; Ding et al., 2009). Endophytic fungi in the genus Fusarium species are reported to produce a diversity of bioactive secondary metabolites including naphthoquinones, e.g., javanicin, fusarubin, solaniol, marticin and nectraiafurone (McLean, 1996; Medentsev and Akimenko, 1998; Thrane, et al., 2004). This class of compounds is of interest due to the broad spectrum of their biological activities, such as antibacterial (Arnstein and Cook, 1947; McCulloch et al., 1982; Baker et al., 1990), antifungal (McCulloch et al., 1982; Tatum et al., 1987), phytotoxic, insecticidal (Claydon et al., 1977) and cytotoxic (Kurobane et al., 1986) properties.

The results from this study are consistent with previous studies indicating the potential of endophytes as producers of cytotoxic agents. Cytotoxic compounds from endophytic extracts have been shown to be active against cervical cancer HeLa (Silva et al., 2006), leukaemia K562 and colon cancer SW116 (Jiao et al., 2006) and non-small-cell lung NCI-H460, pancreatic MIA Pa Ca-2 and CNS glioma SF-268 (Zhan et al., 2007) cancer cell lines. Recently, Phongpaichit et al., (2007) reported 65 endophytic crude extracts from Garcinia species possessed anti-proliferative activity (11.1% against human small-cell lung cancer cell and 12.7% against epidermal carcinoma cell line).
Conclusion

In conclusion, the compound from *Fusarium cf solani* and *Streptomyces* species isolated from *Withania somnifera* and *Datura stramonium* respectively showed a significant cytotoxicity effect on Chang liver cells and HepG-2 cancer cell line. The compounds (3R,5S,10aR)-3,4a,5,6,9,10a-hexahydroxy-7-methoxy-3-methyl-1H,3H,4H,4aH,5H,10H,10aH- naphtho [2,3-c] pyran-10-one (C_{16}H_{20}O_{6}) and Gallic acid were emerging as a promising possible anticancer compound.
Fig 7.1: Untreated Chang Liver Cell; b) Untreated HepG-2 Cell
Fig 7.2: Different concentration of compound isolated from endophytic fungi (WSEPF-3)

a- Treated with 10 μg/ml
b- Treated with 50 μg/ml
c- Treated with 100 μg/ml
d- Treated with 250 μg/ml
Fig 7.3: Different concentration of compound isolated from endophytic actinomycetes (DSEPA-1)

- a- Treated with 10 μg/ml
- b- Treated with 50 μg/ml
- c- Treated with 100 μg/ml
- d- Treated with 250 μg/ml
Fig 7.4: Effect of different concentrations (10 - 250 μg/ml) of C_{16}H_{20}O_{6} isolated from the extracts of endophytic fungi from *Withania somnifera* (WSEPF-3) in Chang liver and HepG-2 cells. Values represent the mean ± SEM of triplicate independent experiments. Cyclophosphamide used as positive control.

Fig 7.5: Effect of different concentrations (10 - 250 μg/ml) of Gallic acid isolated from the extracts of endophytic actinomycetes from Datura stramonium (DSEPA-1) in Chang liver and HepG-2 cells. Values represent the mean ± SEM of triplicate independent experiments. Cyclophosphamide used as positive control.