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PROJECT COMPLETION REPORT

1. **Project Number** : 5136

2. **Title of the Project** : Molecular basis for plumbagin as an antitumor and chemo-sensitizing agent in human breast cancer cells

3. **Funding Agency Name** : DST

4. **Project Reference Number provided by the Funding Agency**: SR/FTP-44/2003

5. **Principal Investigator (Name & Address) :** Dr. G. Srinivas , Scientist D, Department of Biochemistry, SCTIMST, Trivandrum, Kerala

6. **Co-Investigators (Name & Address):** Nil

7. **Implementing Institution(s) and other collaborating Institution** : Rajiv Gandhi Centre for Biotechnology (till May 2004) and Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram.

8. **Date of Commencement** : 24/12/2003

9. **Duration** : 6 years

10. **Date of Completion** : 31/03/2009

11. **Objectives as approved :**
    a) To determine the cytotoxic and apoptotic effects and their cellular mechanisms of plumbagin in various breast cancer cell lines by MTT assay and Thymidine incorporation.
    b) To evaluate the role of hypoxia in modulating the plumbagin's action in breast cancer cells by artificially creating hypoxic conditions.
    c) To study the inhibitory effect of plumbagin in the activation of HIF-1 α in breast cancer cells by western blot analysis.
    d) Decipher the molecular determinants of cell death and chemosensitising effects in plumbagin treated cells.
    e) To study the activation/inhibition of HIF-1 α in breast cancer cells by electrophoretic mobility shift assay.

f) To evaluate and compare all the above properties in primary breast cancer cells with special reference to HIF-1 α.

12. Deviation made from original objectives if any, while implementing the project and reasons thereof :

The project was started at RGCB during the month of January 2004 and reagents and the equipment first sanctioned were purchased during the period till May 2004. During first week of June 2004, the PI shifted his place of work to Department of Biochemistry, SCTIMST, Thiruvananthapuram, where there was no cell culture laboratory existing. The procedures for shifting the project were started immediately. During January 2005 official sanction from DST was granted for the transfer process. However, since the new department did not have the cell culture facility, the work could not be started immediately. The PI through support from his other project funds and institute support managed to establish a cell culture laboratory, including an additional grant from DST to procure carbon dioxide incubator. The lab was partially set during December 2005 and only after that could work actually begin. Accordingly, the objectives set according to the actual plan could not be completely achieved.

The results generated so far were discussed in the GMW held during August 24-25, 2006 at Agharkar Research Institute, Pune and Statement of Expenditure and Utilization Certificate till March 31, 2006 were sent during September 2006. However, there was no reply from DST. The objectives e and f were not completed for the lack of funds from DST. Below is the work done in spite of the difficulties encountered.

13. Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs :

1. **Methodology**:

MTT–cell proliferation assay

This assay was done for cisplatin, taxol and plumbagin as per standard protocol. Human breast cancer cells (MCF-7, SK-Br and HBL-100) were used for the study. Briefly 10,000 cells were seeded into 96 well plate along with DMEM with 10% FCS. After about 24 h, the drugs were added in different concentrations in quadruplicate samples and further incubated for 48 h. The drug-containing medium was replaced with fresh medium with MTT, 5 mg/ml. The formazan crystals formed were solubilized with MTT lysis buffer (20% SDS in 50% dimethyl formamide). Quantitation of color development was done with an ELISA plate reader at 570 nm keeping 630 nm as a reference wavelength.Various combinations of these drugs were tried and their synergistic effects documented.

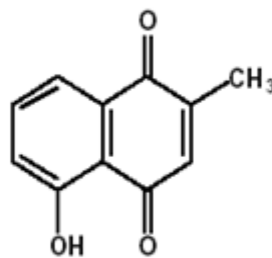
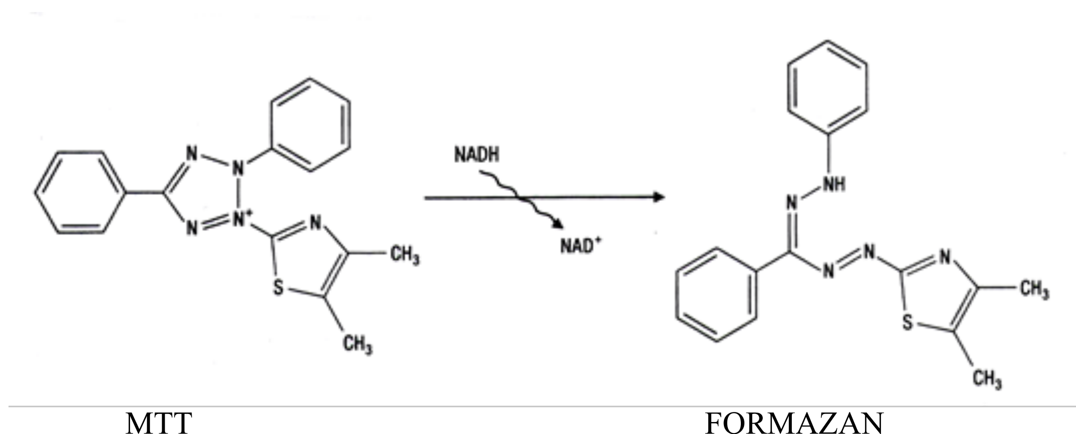
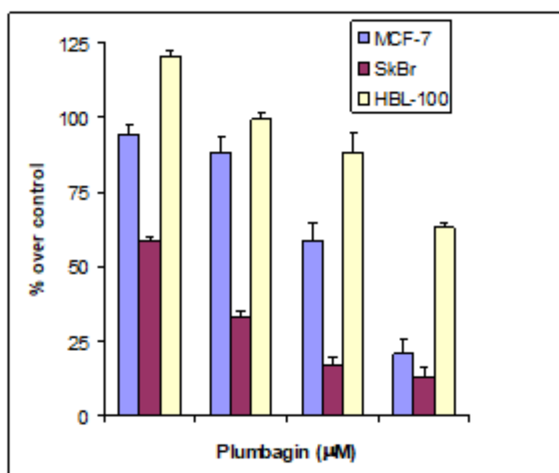


Figure 1- Structure of Plumbagin



MTT FORMAZAN

Figure 2- Molecular structure of MTT and its corresponding reaction product



Cells	LD50 ((μM)
MCF-7	3.75-5.00
SKBr	1.25-2.50
HBL-100	> 5.00

Figure 3- Growth Inhibition Studies on Breast Cancer Cells

Apoptosis Assays

Nuclear Condensation

Loss of cell viability could result from necrosis or apoptosis. Whether the cell death is due to necrosis or apoptosis can be determined on the basis of distinct structural changes in the cell's chromatin, which occurs prior to the lysis of the membrane (Duke and Cohen, 1992). The

changes include extensive condensation of the chromatin, as assessed by light microscopy. Ethidium Bromide is used to evaluate membrane integrity. Acridine Orange will intercalate with the DNA of the cells and will appear bright green. It also binds to RNA and since it cannot intercalate, it appears red-orange. Ethidium Bromide staining is useful to identify non-viable cells.

Annexin-PI staining

As apoptosis causes changes in membrane permeability, there is a flip flop of phosphatidylserine to the outer membrane, which is considered to be one of the early markers of apoptosis. Annexin preferentially binds to phosphatidylserine as it is a negatively charged phospholipid. Hence using FITC conjugated annexin V; apoptotic cells was easily detected with the help of a fluorescent microscope.

TUNEL assay

Briefly, the cells were grown in cover slips and treated with the appropriate concentrations of the compounds with and without the drugs. The cells were washed in PBS and fixed by immersing slides in 4% paraformaldehyde. After washing with PBS, 100 l of TdT reaction were added to the sections on the slide and then incubated at 370C for 60 min inside a humidified chamber for the end labelling reaction to occur. The reactions were terminated by immersing the slides in 2X SSC for 15 min. After washing, endogenous peroxidase activity was blocked by immersing the slides in 0.3% H2O2. After incubation, the colour was developed with the Horseradish-peroxidase-labelled streptavidin and the stable chromogen (Diaminobenzidine). A negative control was also be performed by adding PBS instead of TdT. The slides was then mounted and examined with a light microscope.

Comet assay

For this, the cells after treatment were pelletted and resuspended in 0.5 % low melting point agarose at 370C and layered on a frosted microscope slide previously coated with a thin layer of 0.5 % normal melting agarose and will be kept for 5min at 40C.After solidification, the slides were immersed in lysing solution (2.5 M NaCl, 100mM EDTA, 10mM Tris, 1% triton X-100 and 10% DMSO) for 1h at 40C.After that the slides were electrophoresed for 20 to 60 min at 25 V. The slides after electrophoresis were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide and observed under a fluorescent microscope.

Assessment of Mitochondrial Membrane Potential-$\Delta\psi$m

This assay was done as described earlier (Priya et al., 2004) with ApoAlertTM Mitochondrial Membrane Sensor Kit (Clontech, Palo Alto, CA) for all the 4 compounds. The cells were viewed under confocal laser scanning microscope (488 and 540 nm) and photographed.

Detection of reactive oxygen species

The production of intracellular ROS was measured using dichlorofluroscein diacetate (DCFH-DA) (Chen et al., 1998). This dye is a stable compound that readily diffuses into the cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. ROS generated by the compound oxidizes DCFH to the highly fluorescent compound 2’, 7’-

dichloroflurescein (DCF). Thus the fluorescence intensity is proportional to the amount of peroxide produced. Green fluorescence was be detected by fluorescence microscopy.

14. Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject:

Plumbagin caused cell growth inhibition in breast cancer cells in a time and concentration dependent manner. In the two cancer cell lines studied (MCF-7 and SKBr). The effective concentration needed to kill 50% of the cells was between 2.5-5 M. However, a normal, yet transformed cells line (HBL-100) showed less sensitivity towards plumbagin, which shows the presence of some specificity of this compound towards tumor cells. This finding is only preliminary and needs to be substantiated by drug response to primary cells. Later we carried out combination treatments with standard drugs such as taxol and cisplatin to see whether there exists any augmentation in cell killing with this compound. The results indeed show considerable increase in cell death when cisplatin and plumbagin were used in combination at lower concentration. Cisplatin concentration could be reduced nearly to 25% of the original concentration when used in conjunction with plumbagin.

Analysis of plumbagin induced cell inhibition has lead to the elucidation of apoptosis induction by this compound in breast cancer cells. We had earlier reported induction of apoptosis by plumbagin in cervical cancer cells. There was no clear idea regarding the mechanism of action of this compound at that time. Even now, only few studies are reported analyzing the mode of action of this compound.

In order to establish the mode of action of plumbagin in breast cancer cells, evaluation of apoptosis was carried out. Annexin-PI staining showed clear evidence for apoptosis induction by plumbagin in breast cancer cells. This assay shows the initial stages of apoptosis. Alteration of mitochondirial membrane potential was also documented in drug treated cells, which is believed to be a intermediary step in apoptotic cascade. Comet assay revealed the DNA fragmentation status in these cells. This was further confirmed by TUNEL assay, an alternate method for analyzing DNA fragmentation.

We had earlier shown induction of ROS by plumbagin in cervical cancer cells. Since there could be cell-to-cell variability in modulating the redox potential, we evaluated the formation of ROS in MCF-7 cells. There was a clear indication of ROS generation in MCF-7 cells. The ROS produced thus could be responsible for the observed cytotoxic effect. This is the first report showing the formation of ROS by plumbagin in human breast cancer cells.

Synergistic effects of cisplatin and taxol were also done by MTT assay, which shows that effective cell killing could be achieved through the combined treatment of plumbagin and lower concentration of the standard drugs.

15. Conclusions summarising the achievements and indication of scope for future work :

Plant derived napthaquinone plumbagin had not been subjected to in depth analysis for its possible anticancer property. Earlier reports from BARC reported plumbagin to be an

antioxidant, however, we find that in tumor cells, it is a prooxidant producing ROS. Even though this was speculated earlier due to its quinonid structrure, there was not evidence showing the direct involvement of plumabgin in ROS generation. We had earlier reported this in cervical cancer cells (Srinivas et al 2004). Our study in HBL 100, a transformed, but non-cancer cell line of breast epithelial origin indicated that it is more resistant to plumbagin induced growth inhibition. Moreover combination therapy is getting more acceptable nowadays and this project is an endeavor in providing a background for the hypothesis.

16. Science and Technology benefits accrued :

a. List of research publications with complete details : 2

1. Priya Srinivas, Gokul Gopinath, Asoke Banerji, Abhilash Dinakar, **Gopal Srinivas**. Plumbagin induces reactive oxygen species mediated apoptosis in human cervical cancer cells. ***Mol Carcinog,*** 40(4): 201-211, 2004.
2. **Gopal Srinivas**, Lois A Annab, Gokul Gopinath, Asoke Banerji, Priya Srinivas. Antisense blocking of BRCA1 enhances the sensitivity to plumbagin but not tamoxifen in BG-1 ovarian cancer cells. ***Mol Carcinog***, 39(1): 15-25, 2004.

b. Manpower trained on the project :

- **i. Research Scientists or Research Fellows** : Nil
- **ii. No. of PhD's produced** : Nil
- **iii. Other Technical Personnel trained** : One MSc Student

c. Patents taken, if any : Nil

d. Products developed, if any : Nil

20. Procurement/Usage of Equipment:

a. Details of Equipment:

Sl. No.	Name of Equipment	Make/ Model	Cost (Rs.)	Date of Installa tion	Utilisation	Remarks regarding maintenance breakdown
1	Mini PROTEAN 3 Electrophoresis with Transblot apparatus	Biorad	1 lakh	MAY 2004	90%	Working
2	CO2 Incubator	Sanyo	2.97 Lakhs	Dec 2005	100%	Working

b. Suggestions for disposal of equipment(s): equipment in working condition

(Name and Signature of PI)