Chapter 4
Chapter 4

NOVEL TREATMENT STRATEGIES FOR IMPROVEMENT OF TUMOR CHEMORADIOTherAPY

4.1. INTRODUCTION

4.1.1 Cancer treatment modalities and their limitations

Current cancer therapy modalities comprising surgery, radiotherapy and chemotherapy suffer from limitations of accessibility, targeting, delivery, maintenance of effective doses, toxicity to normal cells and efficacy of the treatment regimen.

Surgery is the oldest form of effective cancer therapy which is used alone or in combination with other modalities. There are multifarious operative risks in cancer patients like comorbid conditions, debilitation associated with cancer, paraneoplastic syndromes, poor nutrition due to anorexia, catabolic influences of tumor growth, neutropenia and thrombocytopenia. In the case of a primary tumor establishing a complete margin of normal tissue is critical for the successful resection of the primary tumor. Primary tumor with local spread sometimes require removal of involved regional lymph nodes and resection of an involved adjacent organ or en bloc resection; and single or a limited number of metastases may require surgical resection of both the primary and metastatic tumor. Radiation therapy, apart from causing damage to the intervening normal tissue and poor healing of surgical wound, can manifest adverse effects such as lethargy, fatigue, mucositis, dermatologic manifestations (erythema, pruritus, desquamation), esophagitis, pneumonitis, hepatitis, gastrointestinal symptoms (nausea, vomiting, diarrhea, tenesmus), genitourinary symptoms (frequency, urgency, dysuria), cytopenias and other late complications like cataracts, keratitis, hypopituitarism, xerostomia, hypothyroidism; pneumonitis, pericarditis, esophageal stricture, hepatitis, ulcers, gastritis, nephritis, sterility, and
muscular contractures etc. (Berger et al. 2002, Gusev et al. 2001, NCRP 2001, Jarrett 1999). Radiation treatment can also increase the risk of developing other malignancies, particularly leukemias and cancers of the thyroid or breast. Traditional chemotherapy agents are designed to block cell division, and therefore can be toxic to healthy as well as cancer cells. However, chemotherapeutic drugs have many adverse effects such as development of drug resistance, drug inactivation by tumor cells, defective apoptosis in tumor cells, bone marrow aplasia, renal failure and hepatic dysfunction. These adverse health effects very often limit the application of anticancer drug at a required concentration.

Improved locoregional control with concurrent chemoradiotherapy is attributed to the better therapeutic benefits of cancer (PreUop and Ove 2005). However, targeting and localized delivery of the drug remain as yet the key challenges in cancer therapy as systemic application of treatment causes severe side effects and in many a time the iatrogenic conditions outweighs the probability of clinical benefit (Jimeno and Cortés-Funes 2005). Therefore targeting specific tumor cell killing by application of biophysical method, radiation and delivering optimum dose of chemotherapeutic drug is chosen as an effective line of therapeutic strategy to stop cancer growth.

4.1.2 Chemoradiotherapy: an approach for effective cancer treatment

Chemoradiotherapy is an approach for cancer treatment by application of combination of chemotherapy and radiation therapy. Despite optimal initial surgery a systemic chemotherapy or radiation therapy is given in some patients to eradicate the residual occult tumor. In some cases chemotherapy and/or radiation therapy are given before surgical resection as neoadjuvant therapy to enhance resectibility and preserve local organ function. Adjuvant based chemotherapy administered with postoperative radiotherapy significantly reduced rates of local recurrence and improved overall survival as compared to surgery alone or in combination with postoperative radiotherapy (Fisher et al. 1988, Krook 1991); However, the most of the
strategies to improve the efficacy of chemoradiation have been failed to significantly improve the outcomes (Glynne-Jones and Debus 2001). Nevertheless, preoperative and concurrent chemoradiotherapy is reported to improve local control and reduce overall treatment associated toxicities (Sauer et al. 2004, Lee et al. 2008).

### 4.1.3 Electrochemotherapy: a novel cancer therapy modality

Electrochemotherapy (ECT) is an exciting new approach to treat certain types of cancer that is proving its efficacy. It combines conventional drug treatment with short high voltage pulsed electric fields to improve the drug delivery. It is an easy, safe and effective treatment of single or multiple nodules of any histology in the cutaneous or subcutaneous tissue. Following systemic administration of anticancer drugs electric field pulses are applied at the target site to ensure maximum killing of the cancer cells. The pulsed electric fields needed for ECT are in the amplitude of a few 100 V/cm to several kV/cm. These fields, though very short, are orders of magnitude higher than other fields associated with low level therapeutic applications. Therefore it is important to localize the fields only to the target tissue in order to minimize the effects to other normal tissues and organs. Application of short electric pulses permeabilizes the cell membrane. This provides chemotherapeutic drugs direct access to the cell cytosol augmenting the cytotoxic effect by several folds.

Reports on ECT studies in different cell lines, animal models and subsequent clinical trials in head and neck squamous cell carcinoma patients (Okino and Mohri 1987, Mir et al. 1988, Mir et al. 1991, Belehradek et al. 1991) showed markedly promising results.

### 4.1.4 Basic principles of electroporation (EP)

Cell EP is a process of transient pore formation in cellular plasma membrane by application of external electric field. Unless the magnitude of generated electrical field exceeds the critical threshold limit, the permeability induced
in the membrane is reversible. Induced pores can remain open from seconds to minutes depending on the type of cell and electrical pulse conditions. For a spherical cell, the conductance of cytoplasm is much higher than that of membrane. As a result of application of external electric field across a cell, membrane potential is generated. The membrane potential ($\Phi_m$) is given as expression: $\Phi_m = 1.5 \ r \ E \ Cos \ \theta$. Where, $E =$ pulse electric field, $r =$ radius of the cell and $\theta =$ angle between the field direction and the radial vector of the surface point and 1.5 represents a constant for the spherical geometry of the cell. Induction of transmembrane potential causes polarization and destabilization of membrane components, which beyond threshold voltage, results in break down at localized areas causing the membrane permeabilization for exogenous molecules (Figure 4.1).
Figure 4.1: Schematic representation of EP

\[ \Phi_m = 1.5 \pi E \cos \theta \]

- High intensity
- Short duration
- Repeatable
4.1.5 EP in drug delivery

Extensive literature on drug delivery systems (Langer 1990, Gregoriadis and Florence 1993, Wallace and Laskar1993) cover mechanism of drug release, advantages of controlled release and problems associated with drug delivery such as limited target access, toxicity to normal/healthy cells, loss of efficacy in the transit etc. Transmembrane transport of smaller molecules takes place by diffusion (Tekle et al. 1990, Tekle et al. 1994, Deleo et al. 1996, Gabriel and Teisse 1999, Neumann et al. 1998) while the transport of macromolecules, particularly DNA (Sukherev et al. 1992, Wolf et al. 1994, Rols and Tiesse 1998, Neumenn et al. 1999) can be accomplished by EP. Studies on Transmembrane transport also imply that electropermeabilization causes uptake of molecule as a function of time, voltage and pulse duration.

Use of EP for anticancer drug delivery to the tumor cells revealed promising results in several studies. Preclinical trials of ECT with bleomycin had shown several hundred fold increase in cytotoxicity at the level of 50% cell kill by EP (Gehl et al. 1998, Orlowski et al. 1988). Similarly cisplatin has been tried for treatment of melanoma (Sersa et al. 2000, Sersa et al. 1998, Gothelf et al. 2003). Radiation toxicity and cisplatin induced toxicity were found enhanced by EP in vivo (Chemazar et al. 2001). Recent research has involved the use of EP to enhance the radiation effects leading to different mode of therapeutics (Shil et al. 2005).

4.1.6 Scope and objectives

Lack of specificity of conventional external beam radiation and chemotherapy (Vaidyanathan and Zalutsky 1996) and resistance of tumor cells (Pandey and Mishra 1999) often prevent application of these treatments at potentially curative levels because of supervening toxicities to normal tissues. It is believed that lack of effective cellular concentration of drugs fails to kill the cancer cells and presence of hypoxic regions in the solid tumor mass evokes poor response to radiation treatment. Intensive efforts
are needed to increase the effectiveness of cancer therapy through a number of strategies to improve the therapeutic index.

Over the past years considerable attention has been given to targeting the “hallmarks” (Hanahan and Weinberg 2000) of cancer such as self-sufficiency in growth signals, insensitivity to antigrowth signals, evading cell death or apoptosis, limitless replicative potential, angiogenesis and tumor invasion and metastasis. However, despite improvements in survival rates, cancer remains the leading cause of death in the world. Therefore, for improvement of effective drug delivery and enhancement of radiation response, EP could be used as a combinatorial strategy for improvement of cancer therapy.

In the previous chapter we have observed that porphyrin analoges, 5,10,15,20- tetrakis [3,4-bis (carboxymethyleneoxy) phenyl]-porphyrin (DHBEPH) and meso-5,10,15,20- tetrakis [4-carboxymethyleneoxy-phenyl] porphyrin have shown significant uptake in transplanted thymic lymphoma (TL) and fibrosarcoma tumors in mice. Subsequently a radioisotope based pre-therapeutic diagnostic protocol using $^{177}$Lu-labeled Porphyrin-p-NH$_2$-benzyl-DOTA conjugate was described for TL tumor in mouse model. After diagnosis, we evaluated a novel targeted radionuclide therapy using porphyrin derivatives as an effective tumor treatment modality.

It was our attempt to study the efficacy of an easy and less toxic treatment strategy. We used EP with a view to explore its feasibility of use in combination of drug and radiation which might have relevance in developing a preferred clinical approach for dealing with superficial or recurring tumor not easily amenable to conventional treatment. Present work was designed to enhance cancer cell cytotoxicity in vitro by using EP in combination with chemotherapeutic drug and γ-irradiation. The study was then extended to in vivo TL tumor in mice. The efficacy of combination therapy of chemotherapeutic drug, γ irradiation and EP was compared with the single treatment modality.
### 4.2 MATERIALS AND METHODS

#### 4.2.1 Tumor and cell lines

Fibrosarcoma, an weakly immunogenic tumor was originally developed by administration of dimethylbenzdithionaphene (DBDN) in syngenic mice. The Ehrlich’s ascites carcinoma (EAC) cell line is a transplantable malignant tumor, which was originally developed from a spontaneous breast cancer in mouse. The thymic lymphoma (TL) was a transplantable tumor developed by the author and his coworker in his laboratory from radiation-induced thymic lymphoma tumor. All the tumor cell lines were maintained in vivo in the female Swiss mice (6-8 wks) by serial transplantation. EAC cells were maintained in the peritoneal cavity and the fibrosarcoma and TL tumors were maintained either subcutaneously on the dorsum or intramuscularly in the thigh muscles of the animal.

#### 4.2.2 Antineoplastic drugs

Cyclophosphamide (Khandelwal Laboratories Ltd., Mumbai, India) at concentrations of 1.25 mg, 2.5 mg, 5.0 mg and 7.5 mg; Vinblastin sulphate (Cipla Ltd, Mumbai, India) at concentrations of 0.025 mg and 0.05 mg and Dox hydrochloride (Pharmacia & Upjohn S.P.A, Italy) at concentrations of 0.125 mg and 0.25 mg were added to TL tumor cell suspension in sterile normal saline solution containing $1\times10^6$ cells/0.2 ml and injected subcutaneously to each of the normal mice of ten in each dose group. For tumor growth inhibition studies doxorubicin (Dox) at the doses of 0.125 mg (~6.25 mg/kg) and 0.25 mg (~12.5 mg/kg) were administered i.v. to the mice bearing TL tumor.
4.2.3 Animals, irradiation and preparation of thymic lymphoma (TL) cell suspension

Inbred Swiss mice bred and reared in the laboratory animal facility of Bhabha Atomic Research Centre, Mumbai, India, were used in the studies as mentioned in Chapter 2 (Section 2.2.1). Mice bearing tumor were immobilized in specially designed well ventilated perspex holder with lead shield (~ 5 mm thick) and the tumor was locally irradiated with 2 Gy (at a dose rate of 0.39 Gy/min) using a Junior Theratron (MDS Nordian, Canada) teletherapy machine.

To obtain the tumor cell suspension, animals were sacrificed with overdose of ether anesthesia. Tumor tissues obtained were used to prepare the cell suspension as mentioned in Chapter 2 (Section 2.2.9). The tumor cells were suspended either in PBS or in isotonic sucrose solution (272 mM, pH: 7.4). The cells were exposed to a desired radiation dose of 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16 and 20 Gy of γ-rays at a dose rate of 0.39 Gy/min at room temperature by using the same radiation source as mentioned above.

4.2.4 Trypan blue dye exclusion test for cellular viability

Trypan blue, a cationic chromophore that intercalates to DNA was used to check cellular viability as mentioned in Chapter 2 (Section 2.2.11)

4.2.5 Electroporation (EP) parameters

A Medical Electroporator (Model BARC/RB&HSD/01-04) developed in-house was used for application of EP at a desired field strength of 0, 0.5, 1, 1.5, 2, 2.5 or 3 kV/cm. Monophasic rectangular electric pulses of 10 bursts (each burst having 8 number of pulses with 200 μs pulse duration) were applied to the tumor cells in vitro and transplanted tumors in vivo. EP chambers and electrodes for in vitro (Figure 4.2) and electrodes for in vivo (Figure 4.3) studies were designed indigenously. For in vitro application of EP, tumor
cells were suspended in EP medium (9.31 g sucrose in 100 ml d/w, pH 7.4) and transferred to the EP chambers. Flat parallel plate steel electrodes of 2 cm x 2 cm size were mounted on a vernier calipers for application of electric pulses to the tumor transplanted on the thigh region of Swiss mouse. The electrodes were placed at the opposing margins of the tumor percutaneously by adjusting their position according to the size of the tumor (Figure 4.3). For ensuring good contact between the skin and the electrodes and reducing the resistance of the skin a conducting gel (Electrode gel®, Technomed, Mumbai, India) was used prior to the application of electric pulses.

A sample volume of 0.4 ml was used in the EP chamber (cuvette) with parallel plate metal electrodes. Conductivity of PBS was much higher (37.2 mho) than sucrose (272 mM) solution (18 μho) and therefore PBS offered very low resistance compared to the output impedance of the electroporator. Since sucrose solution offered higher resistance and thereby higher amplitude of applied pulses experiments wherever appropriate were conducted on sucrose medium.

A few initial experiments on EAC cells in vitro were the extension of the previous worker (Shil 2006) in the laboratory.
Figure 4.2: In-house designed electroporator chamber (cuvette) with flat parallel electrodes for application of electric pulse to the tumor cells in suspension

Figure 4.3: Application of electric pulse on the TL tumor transplanted in the right thigh of a Swiss mouse. Parallel plate electrodes designed in-house and mounted on a vernier calipers were placed in orthoposition of the tumor for application of monophasic rectangular pulses
A sample volume of 0.4 ml was used in the EP chamber (cuvette) with parallel plate metal electrodes. Conductivity of PBS was much higher (37.2 mho) than sucrose (272 mM) solution (18 µho) and therefore PBS offered very low resistance compared to the output impedance of the electroporator. Since sucrose solution offered higher resistance and thereby higher amplitude of applied pulses experiments wherever appropriate were conducted on sucrose medium.

A few initial experiments on EAC cells in vitro were the extension of the previous worker (Shil 2006) in the laboratory.

4.2.6 Measurement of intracellular ROS in tumor cells using H$_2$DCFDA as fluorescent probe

Generation of ROS in tumor cells treated with $\gamma$-irradiation, EP and anticancer drug, Dox was estimated by fluorescent probe, 2',7'-Dichlorofluorescein diacetate (H$_2$DCFDA) (Molecular probes, USA). Briefly, cell suspension (1x10^6 cells / ml of PBS or 272 mM sucrose solution) was incubated with H$_2$DCFDA (10 µM in ethanol, 0.001 v/v) at 25 °C for 20 min prior to the treatments. Fluorescence intensity was measured in the different samples using spectrofluorimeter (LS50B, Perkin Elmer, USA) with $\lambda_{ex} = 488$ nm and $\lambda_{em} = 520$ nm, bandwidth excitation/emission = 5 nm/10 nm respectively.

4.2.7 Detection of apoptosis in cancer cells using annexin V

Apoptotic index in control and treated cancer cells was determined by annexin-V-fluorescence kit (Roche, Germany) following the protocol provided along with the kit. Briefly, 50 µl annexin V solution was added to the cell pellet of ~1x10^6 cells per sample, incubated for 15 min at 25 °C and the cells were observed under fluorescence microscope (Optiphot 2, Nikon, Japan). Out of 400 number of cell taken randomly from the microslide, green or red cells were counted severally. Cells showing both red and green fluorescence were considered necrotic. Percentage apoptosis or apoptotic index (AI) was
calculated as the ratio between green and the total cells multiplied by 100. Phosphatidylserine (PS) localized in inner leaflet of the membrane bilayer in normal cells flips to the outer leaflet in apoptotic cells. Annexin V, a glycoprotein tagged with fluorescein, having specificity for PS binds only to apoptotic cells giving green fluorescence ($\lambda_{ex} = 490$ nm and $\lambda_{em} = 520$ nm). The dye is added with PI which gives red fluorescence and distinguished between apoptotic and non-apoptotic cells.

4.2.8 Transplantation and measurement of growth kinetics of TL tumor

Single cell suspension of $2 \times 10^5$ cells in 0.15 ml volume were transplanted to quadriceps femoris muscle in the right hind limb of female Swiss mice. Tumor diameters were measured along the two mutually orthogonal axes of the transplanted tumor using a digital vernier caliper (Mitutoyo, Japan). Volume of tumor was calculated using following formula:

$$\text{Tumor volume (mm}^3) = ab^2 \left(\frac{\pi}{6}\right) \text{ (Heller et al. 1995, Kenji et al. 2000)}$$

where, $a$ stands for the largest diameter (mm) and $b$ stands for the diameter perpendicular to $a$.

4.2.9 Combination treatment of radiation, doxorubicin (Dox) and EP

On 9th day of transplantation, mice with palpable tumors were divided randomly into 5 groups: i) control (no treatment), ii) radiation (2 Gy of $\gamma$-irradiation), iii) Dox (0.5 mg/kg b wt.), iv) EP (2kV/cm) and v) combination therapy (radiation + Dox+ EP) consisting 7 animals in each group. For combination therapy, right hind limbs of mice bearing TL tumor were locally irradiated with 2 Gy of $\gamma$-rays. Subsequently the animals were administered with 10 $\mu$g of Dox and within 3-4 minutes monophasic rectangular electric pulses of 10 bursts (with 8 pulses/burst, pulse duration 200 $\mu$s) were applied to the tumor at a field strength of 2 kV/cm. The tumor measurement was taken daily up to 7 days following the treatments.
4.2.10 Tumor volume, doubling time (DT) and specific growth rate (SGR) measurement

Measurement of tumor volume, DT and SGR after different treatment conditions were done as mentioned in Chapter 3 (Section 3.2.5)
4.3 RESULTS

4.3.1 Viability of thymic lymphoma (TL) tumor cells in sucrose solution

TL tumor cells did not show much variation in viability in the sucrose (272 mM, pH 7.4) solution incubated for 0, 60, 120 and 180 min at 37 °C indicating suitability of the solution for use in electroporation (EP) and γ-irradiation (Figure 4.4). The variation in per cent viability due to incubation time ranged from 95.83±0.42 to 91.93±0.35 at 0 and 180 min of incubation respectively.

![Viability of TL tumor cells in EP medium at 37 °C at different incubation time. The viability was evaluated by Trypan blue dye exclusion method](image)

Figure 4.4: Viability of TL tumor cells in EP medium at 37 °C at different incubation time. The viability was evaluated by Trypan blue dye exclusion method

4.3.2 Effect of radiation and EP on Ehrlich’s ascites carcinoma (EAC) cells

EAC cells were exposed to γ-irradiation (0-10 Gy) and EP (0-3 kV/cm) to study the cytotoxic effect of the treatments (Figure 4.5 A & B). The toxic
effect was prominent at longer incubation period i.e. 3 h than at 1 h. Percentage (%) viability of the cells decreased from 91.70 ± 0.56 to 68.87 ± 1.27 in the 0 and 10 Gy exposed cells respectively at 3 h post incubation at 37 °C (Figure 4.5 A). Similarly increasing strength of applied electric field reduced the percentage viability of the cells from 94.77 ± 1.27 to 49.20±2.09 in 0 kV/cm and 3 kV/cm respectively at 1h post-EP. The cytotoxic effect was further enhanced up to 61.94 % when incubation period was extended to 3 h after application of EP at field strength of 3 kV/cm (Figure 4.5 B).

![Figure 4.5](image)

**Figure 4.5:** Cellular viability of Ehrlich's ascites carcinoma cells exposed to 0-10 Gy of 60Co γ-irradiation (A) and electroporation (B). The viability was evaluated by Trypan blue dye exclusion method at 1 and 3 h of incubation

### 4.3.3 Effect of EP on radiosensitivity of EAC cells

EAC cells in sucrose solution were exposed to 3 Gy 60Co γ-radiation alone or in combination with monophasic electric pulses at a field strength of 2.5 kV/cm and viability was measured at 1 and 3 h post-exposure (Figure 4.6). There was a decrease in percent cell viability in treated groups and the decrease was in the order of control< irradiation< EP < irradiation + EP.
Viability of the cells was also found decreased with the increasing post-treatment incubation time. Combination treatment of radiation and EP exerted maximum effect to the cells as evidenced from the decreased percentage viability from 95.13±0.38 in control to 71.9±2.41 and 59.57±0.78 after 1 and 3 h incubation respectively.

![Figure 4.6](image)

**Figure 4.6:** Effect of γ-irradiation (3Gy) and electroporation at a field strength of 2 kV/cm (EP) and in combination (3Gy+EP) on viability of Ehrlich ascites carcinoma cells. Cell viability was evaluated by Trypan blue dye exclusion method after 1 and 3h of incubation following the treatment

### 4.3.4 Effect of EP on radiosensitivity of fibrosarcoma cells

Fibrosarcoma cells in sucrose solution were exposed to monophasic electric pulses of increasing field strength in combination with 3 Gy ^60^Co gamma radiation and viability was measured 3 h post exposure (Figure 4.7). A decrease in viability of the tumor cells was observed with increasing field strength of EP alone or in combination with radiation exposure given either

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before or after EP. Radiation treatment given before the application of EP was more effective in terms of enhancing the tumor radio-cytotoxicity.

**Figure 4.7:** Effect of EP at field strength of 0.0-3.0 kV/cm and 60Co γ-irradiation (3Gy) either after (EP+3Gy) or before (3Gy+EP) on viability of fibrosarcoma cells. EP was carried out within 10 min of irradiation. Cell viability was evaluated by Trypan blue dye exclusion method after 3 h of EP.

### 4.3.5 Sensitivity of TL tumor cells to combination treatment of radiation and EP

Cytotoxic effect of γ-irradiation and EP on TL tumor cells was studied by exposing the cells to 0-10 Gy of 60Co γ-rays (Figure 4.8), electric pulses of 0-3 kV/cm field strength (Figure 4.9) and a combination of 3 Gy of γ-irradiation and electric pulses of 2 kV/cm field strength (Figure 4.10). The cytotoxic effect was enhanced with the increasing radiation doses, field strength of EP and post-treatment incubation time. Marked decrease in viability was
observed in case of both radiation and EP treatment of the cancer cells after 3 h incubation.

At 3 and 10 Gy of irradiation the decrease in cell viability was 6.21 and 18.08 % respectively after 1 h which was further increased to 7.48 and 34.35% respectively after 3 h incubation. Similarly, at 2 and 3 kV/cm EP the decrease in the cell viability was 13.81 and 46.30 % respectively at 1 h, which was enhanced to 17.18 and 69.07% respectively at 3 h incubation. When the cancer cells treated with 3 Gy followed by electric pulses (2 kV/cm), the viability was further reduced to 28.37 % as against 7.48 and 17.18 % reduction achieved by the respective treatments alone.

![Figure 4.8: Viability of TL tumor cells treated with 0, 1, 3, 5, 8 and 10 Gy of 60Co γ-rays at 37 °C. The viability was evaluated by Trypan blue dye exclusion method at 1 and 3 h incubation following irradiation](image)
Figure 4.9: Cellular viability of TL tumor cells exposed to electroporation (EP) of increasing field strength (0.0-3.0 kV/cm) The viability was evaluated by Trypan blue dye exclusion method at 1 and 3 h of incubation following EP.

Figure 4.10: Effect of γ-irradiation (3 Gy) and electroporation at a field strength of 2 kV/cm (EP) and in combination on viability of thymic lymphoma (TL) tumor cells. The viability was evaluated by Trypan blue dye exclusion method at 0 and 3 h of incubation following exposure to EP.
4.3.6 Intracellular ROS generation following treatment of TL tumor cells with radiation and EP

In order to understand the role of intracellular ROS in cytotoxicity of TL tumor cells, H$_2$DCFDA fluorescence was measured by exposing the tumor cells to $^{60}$Co $\gamma$-rays (0-10 Gy) and EP (0-3 kV/cm) or in combination of 3 Gy of $\gamma$-rays and 2 kV/cm field strength. There was a dose dependent increase in generation of intracellular ROS with respect to the applied radiation doses (Figure 4.11) and electric fields (Figure 4.12). The increase in ROS generation was more prominent in case of $\gamma$-irradiation. The generation of ROS as determined in terms of H$_2$DCFDA fluorescence was higher by 2.38 and 5.24 fold with 3 and 10 Gy irradiation and 1.81 and 2.23 fold with 2 and 3 kV/cm field strength respectively. However, the increase in ROS was 2.72 fold when tumor cells were given combined treatment of 3 Gy irradiation and EP at 2 kV/cm field strength (Figure 4.13).

![Figure 4.11: ROS generation in TL tumor cells following exposure to different doses of $^{60}$Co $\gamma$-rays measured in terms of H$_2$DCFDA fluorescence. The cells ($1\times10^6$/ml) in PBS were labeled with H$_2$DCFDA (13 mM) and fluorescence was measured at 520 nm. Data shown are from a representative experiment in triplicates (Mean ± SD)](image)

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Figure 4.12: ROS generation in TL tumor cells following electroporation (EP) treatment (0.0-3.0 kV/cm) and measured in terms of H$_2$DCFDA fluorescence. The cells (1x10$^6$/ml) in PBS were labeled with H$_2$DCFDA (13 mM) and fluorescence was measured at 520 nm. Data shown are from a representative experiment in triplicates (Mean ± SD).

Figure 4.13: ROS generation in TL tumor cells following exposure to $^{60}$Co γ-rays (3 Gy), electroporation at a field strength of 2 kV/cm (EP) and a combination of the both (3 Gy + EP) and measured in terms of H$_2$DCFDA fluorescence. The cells (1x10$^6$/ml) in PBS were labeled with H$_2$DCFDA (13 mM) and fluorescence was measured at 520 nm. Data shown are from a representative experiment in triplicates (Mean ± SD).
4.3.7 Cytotoxicity, apoptosis and ROS generation in TL tumor cells treated with Dox and EP

Cytotoxicity of TL tumor cells due to treatment with Dox and EP was measured. The tumor cells were treated with Dox (5 or 10 μg) and EP (at a field strength of 2 kV/cm) or in combination of Dox and EP. Enhanced cytotoxic effect was observed with the increasing dose of Dox and post-treatment incubation time.

Dox at 5 and 10 μg concentration decreased the percentage viability of TL tumor cells by 7.98 and 10.67 % respectively at 0 h of incubation. With the increase in the incubation time to 3 h the viability was decreased to 16.23 % in 10 μg treated cells without changing the viability at 5 μg concentration. Treatment of the cells with EP (2 kV/cm) brought down the mean percentage viability by 9.86 and 18.62 % at 0 and 3 h post-incubation respectively.

However, Dox in combination with the EP worked synergistically to enhance the cytotoxicity markedly in the TL tumor cells. The combination treatment decreased the percentage viability of the cells by 26.0 and 43.40 % at 5 μg and 44.34 and 69.99 % at 10 μg of Dox at 0 and at 3 h post-incubation respectively (Figure 4.14).
Figure 4.14: Effect of doxorubicin at concentrations of 5 μg (Dox 5 μg) and 10 μg (Dox 10 μg), electroporation at a field strength of 2 kV/cm (EP) alone and combination of EP with Dox 5 μg (Dox 5 μg+EP) and 10 μg (Dox 10 μg+EP) on viability of TL tumor cells. Viability was evaluated by Trypan blue dye exclusion method at 0 h and 3 h of incubation following the treatments. Data shown are from a representative experiment in triplicates (Mean ± SD) from a set of three.

Induction of apoptosis in TL tumor cells was evident after the cells were treated with Dox and EP (Figure 4.15). Apoptotic index was increased in the tumor cells due to the drug and EP treatments and at longer incubation period. The increase in apoptosis was in the order of control <Dox 5 μg <EP <Dox 10 μg <Dox 5 μg and EP <Dox 10 μg and EP. Exposure to EP (2 kV/cm) and Dox (5 μg) enhanced apoptotic index to 9.75 at 3 h post-incubation. Percentage apoptosis was increased 3 fold with increased dose of Dox (10 μg). When the Dox doses of 5 and 10 μg were combined with the EP the mean apoptotic index was enhanced to 5.68 and 8.3 fold respectively at 3 h post-incubation.
Figure 4.15: Effect of doxorubicin at concentrations of 5 μg (Dox 5 μg) and 10 μg (Dox 10 μg), electroporation at a field strength of 2 kV/cm (EP) alone and combination of EP with Dox 5 μg (Dox 5 μg+EP) and 10 μg (Dox 10 μg+EP) on induction of apoptosis in TL tumor cells. Apoptosis in the tumor cells were determined by annexin V fluorescence. Data shown are (Mean ± SD) from a set of three experiments.

Figure 4.16 showed the cellular oxidative stress in terms of ROS generation due to treatment of TL tumor cells with Doxorubicin and EP. Intracellular ROS generation shown as H2DCFDA fluorescence was increased in the order of the treatments, EP, Dox 5 μg, Dox 10 μg, EP and Dox 5 μg and EP and Dox 10 μg compared to control. ROS generation in case of EP (2 kV/cm) or Dox 5 μg treatment alone was comparable. The increase in fluorescence intensity was 1.43 and 1.54 folds respectively for both the treatments. Treatment of the tumor cells with Dox 10 μg increased the fluorescence intensity by 2.13 fold over control. Similar or better results (2.39 fold increase) could be achieved with 5 μg of Dox when treated in combination
with the EP. Combined treatment of Dox at the dose of 10 μg with EP however resulted in maximum (2.64 fold) increase in fluorescence intensity.

**Figure 4.16:** Effect of doxorubicin at concentrations of 5 μg (Dox 5 μg) and 10 μg (Dox 10 μg), electroporation at a field strength of 2 kV/cm (EP) alone and combination of EP with Dox 5 μg (Dox 5 μg+EP) and 10 μg (Dox 10 μg+EP) on intracellular ROS generation in TL tumor cells. ROS generation was measured in terms of H2DCFDA fluorescence. Cells (1x10⁶/ml) in PBS were labeled with H2DCFDA (13 mM) and fluorescence was measured at 520 nm. Data shown are from a representative experiment in triplicates (Mean ± SD)

### 4.3.8 Growth delay and inhibition in TL tumor induced by γ-irradiation of transplanted tumor cells

#### 4.3.8.1 Growth delay in TL tumor induced by γ-irradiation

Table 4.1: showed results of the experiments on growth delay in the TL tumor in female Swiss mice (6-8 wks) following transplantation of in vitro irradiated (0, 2, 4, 8, 12, 16, 20 Gy) tumor cells (3x10⁵ cells/animal). The growth delay in the tumor was evaluated in terms of appearance on post-transplantation days. There was no visible change in tumor appearance
pattern in animals receiving 2 and 4 Gy irradiated cells compared to control. With the dose of 8 Gy tumor appearance was reduced and delayed as well. The appearance of the tumor was further delayed up to 15 d when transplanted tumor cells were exposed to 12 and 16 Gy of γ-radiation. The appearance of the tumors on 21 d was 80, 80 and 60 % in the animals receiving the tumor cells exposed to 8, 12 and 16 Gy irradiation respectively. The cells receiving irradiation of 20 Gy did not produce any tumor in the mice.

**Table 4.1:** Appearance of tumor in female Swiss mice (6-8 wks) on 9, 12, 15, 18 and 21 d following transplantation of in vitro γ-irradiated TL tumor cells

<table>
<thead>
<tr>
<th>Rad dose (Gy)</th>
<th>Appearance of tumors (post-transplantation days)</th>
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<tr>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

Tumor cells (3x10^5) in suspension were injected s.c. at the dorsum of each mouse, (n=10).

**4.3.9.2 Growth inhibition in TL tumor induced by γ-irradiation**

The percent growth inhibition of the TL tumor (Table 4.2) was significantly increased with pre-exposure of the transplanted tumor cells to increasing doses of radiation. The tumor growth was inhibited by 7.73, 13.56 and 36.29 % corresponding to 2, 4 and 8 Gy exposure respectively on 12 d of transplantation.
Table 4.2: Inhibition of growth of tumor in female Swiss mice (6-8 wks) following transplantation of in vitro γ-irradiated TL tumor cells

<table>
<thead>
<tr>
<th>Radiation dose (Gy)</th>
<th>Tumor volume in mm³ (post-transplantation days)</th>
<th>% growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(9 d)</td>
<td>(10 d)</td>
</tr>
<tr>
<td>0</td>
<td>302.45 ±121.46</td>
<td>882.57 ±511.90</td>
</tr>
<tr>
<td>2</td>
<td>283.18 ±136.82</td>
<td>822.90 ±372.07</td>
</tr>
<tr>
<td>4</td>
<td>252.40 ±142.30</td>
<td>671.66 ±292.41</td>
</tr>
<tr>
<td>8</td>
<td>178.50 ±76.07</td>
<td>448.60 ±273.05</td>
</tr>
</tbody>
</table>

Tumor cells (3×10⁶) in suspension were injected s.c. at the dorsum of each mouse, (n=10). The values are Mean± SD.

4.3.9 Appearance of TL tumor in presence of antineoplastic drugs

In the previous experiments the TL tumor cells were shown to be sensitive to an in vitro irradiation of 20 Gy. These TL tumor cells resembled non-Hodgkin’s lymphomas (NHL) which in human are generally treated with a combination chemotherapy, CHOP (cyclophosphamide, Dox hydrochloride [formerly hydroxyldaunomycin], vincristine sulfate [Oncovin] and prednisone). Therefore, the chemosensitivity of the TL tumor cells to cyclophosphamide, vinblastin and doxorubicin hydrochloride (Dox) was investigated in terms of tumor growth inhibition.

Cyclophosphamide at concentrations 1.25 and 2.5 mg caused appearance of tumor in 20% of mice within 30 days. However, during this period, growth of the tumor was not detected at the dose of 5 mg which is calculated to be about 6 times higher than the human therapeutic dose rate on per kg body weight basis. At the dose of 7.5 mg 80% of the mice died within 15 days without any sign of tumor growth. The remaining 20% of animal which survived at this dose tumor was not visible up to 30 days. Tumors were developed in all ten mice in the concurrent control group (Table 4.3).
**Table 4.3:** Effect of cyclophosphamide on the growth of TL tumor

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>No. of mice treated</th>
<th>Tumor incidence on 30 d (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>10 (100)</td>
</tr>
<tr>
<td>1.25</td>
<td>10</td>
<td>2 (20)</td>
</tr>
<tr>
<td>2.5</td>
<td>10</td>
<td>2 (20)</td>
</tr>
<tr>
<td>5.0</td>
<td>10</td>
<td>0 (0)</td>
</tr>
<tr>
<td>7.5</td>
<td>10</td>
<td>0 (0)*</td>
</tr>
</tbody>
</table>

Cyclophosphamide was added to 0.2 ml of tumor cells (1x10⁶) suspension in normal saline and injected subcutaneously to each mouse. * Eight animals died within 15 days without sign of tumor development.

Administration of vinblastin at a dose of 0.025 mg did not affect the development of tumor. At the dose of 0.05 mg tumor was developed in 60% of mice within 40 days against 100% tumor incidence in the untreated control group (Table 4.4).

**Table 4.4:** Effect of vinblastin on the growth of TL tumor

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>No. of mice treated</th>
<th>Tumor incidence on 40 d (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>10 (100)</td>
</tr>
<tr>
<td>0.025</td>
<td>10</td>
<td>10 (100)</td>
</tr>
<tr>
<td>0.05</td>
<td>6</td>
<td>6 (60)</td>
</tr>
</tbody>
</table>

Vinblastin was added to 0.2 ml of tumor cells (1x10⁶) suspension in normal saline and injected subcutaneously to each mouse.

Dox added to the TL tumor cell suspension at concentrations of 0.125 and 0.25 mg/1x10⁶ cells and injected s.c. to the female Swiss mice exerted its cytotoxic effect revealed by non-appearance of the tumor up to 60 d (Table 4.5).
Table 4.5: Effect of doxorubicin (Dox) on TL tumor cells

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>No. of mice treated</th>
<th>Tumor incidence on 60 d (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>0.125</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Dox was added to 0.2 ml of tumor cells (1x10⁶) suspension in normal saline and injected subcutaneously to each mouse.

4.3.9.1 Tumor growth inhibition by Dox

Dox at a concentration of 0.125 mg could completely inhibit the growth of the TL tumor whenever the drug was added to the tumor cell suspension at the time of transplantation to the host. Therefore in the next experiment it was designed to administer the drug systemically (i.v.) to the tumor bearing mice. Dox when injected to the mice bearing small sized (0.004 cm³) tumor at the dose of 0.125 mg, tumor disappeared in 40% of the mice. The tumor was completely regressed in all the mice when Dox dose was increased to 0.25 mg. However, Dox when injected to the mice bearing relatively larger (0.09 cm³) tumor, the growth of the tumor was significantly inhibited (0.16 ± 0.12 cm³) on 3 d as compared to control (1.39 ± 0.14 cm³) and 0.125 mg Dox treated group (1.62 ± 0.32 cm³) (Table 4.6). The results indicated greater effectiveness of Dox at the dose of 0.25 mg in combating the growth of TL tumor.
Table 4.6: Tumor growth inhibition by doxorubicin (Dox) in TL bearing mice

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>No. of mice treated</th>
<th>Tumor volume (cm³)</th>
<th>0 d</th>
<th>3 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td></td>
<td>0.09 ± 0.04</td>
<td>1.39 ± 0.14</td>
</tr>
<tr>
<td>0.125</td>
<td>10</td>
<td></td>
<td>0.09 ± 0.04</td>
<td>1.62 ± 0.32</td>
</tr>
<tr>
<td>0.25</td>
<td>10</td>
<td></td>
<td>0.07 ± 0.03</td>
<td>0.16 ± 0.12</td>
</tr>
<tr>
<td>0.125</td>
<td>10</td>
<td></td>
<td>0.004</td>
<td>Tumor regressed in 40%</td>
</tr>
<tr>
<td>0.25</td>
<td>6</td>
<td></td>
<td>0.004</td>
<td>Tumor regressed in all</td>
</tr>
</tbody>
</table>

Dox was administered i.v. to female Swiss mice on 5 or 9 d post-transplantation of tumor. The volume of the tumor was Mean±SEM.

4.3.10 Inhibition of TL tumor growth kinetics by radio-electrochemotherapy

Figure 4.17: showed the growth kinetics of TL tumor in female Swiss mice. The average tumor volume in control animals on 1 and 7 d was 41.54 ± 40.80 and 2370.70 ± 346.63 mm³ respectively. The tumor volume in the radiation (2 Gy) treated group was 40.79 ± 13.84 and 2570.65 ± 485.29 mm³ on 1 and 7 d; EP (2kV/cm) treated group were 44.57 ± 13.87 and 2725.35 ± 219.32 mm³; and in Dox (10 µg) treated group was 34.06 ± 15.79 and 1967.04 ± 278.94 mm³ on the corresponding days. The average tumor volume in the animals treated with the combination therapy i.e. radiation, Dox and EP on 1 and 7 d were 37.77 ± 15.70 and 1235.58 ± 97.21 mm³, respectively. The result showed that with single treatment of radiation (2 Gy) or Dox (10 µg) there was increase in average tumor volume by 8.43 and 17.03 % compared to control on 7 d of treatment. However, with EP (2kV/cm) the average tumor volume reduced to 17.03 %. The reduction in tumor volume with the combination treatment was maximum (47.88%) on the 7 d of treatment. However, there was not much difference in tumor...
doubling time (DT) in the control and the treated groups (Table 4.7). The DT in TL bearing mice was 1.18 ± 0.14 d in the combination therapy group as against 1.03 ± 0.06 d in the control. The specific growth rate (SGR) of the tumor was found 0.68 ± 0.04, 0.70 ± 0.06, 0.69 ± 0.06, 0.69 ± 0.08, and 0.59 ± 0.08 %/d in control, radiation, Dox, EP, and combined therapy (Rad +Dox +EP) groups respectively.

**Figure 4.17**: Growth kinetics of TL tumor in Swiss mice following treatment with 2 Gy of $^{60}$Co γ-irradiation (Rad), 10 μg of doxorubicin (Dox), EP at a field strength of 2 kV/cm (EP), and a combination of the γ-irradiation, Dox and EP (Rad +Dox +EP)
Table 4.7: Tumor doubling time (DT) and specific growth rate (SGR) of TL tumor in female Swiss mice following treatment with 2 Gy of $^{60}$Co $\gamma$-irradiation (Rad), 10 $\mu$g of doxorubicin (Dox), EP at a field strength of 2 kV/cm (EP), and combination of the $\gamma$-irradiation, doxorubicin and EP (Rad + Dox + EP)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DT (d)</th>
<th>SGR (%/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.03 ± 0.06</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td>Rad</td>
<td>1.00 ± 0.08</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>Dox</td>
<td>1.01 ± 0.09</td>
<td>0.69 ± 0.06</td>
</tr>
<tr>
<td>EP</td>
<td>1.01 ± 0.12</td>
<td>0.69 ± 0.08</td>
</tr>
<tr>
<td>Rad+Dox+EP</td>
<td>1.18 ± 0.14</td>
<td>0.59 ± 0.08</td>
</tr>
</tbody>
</table>
In the previous chapter, effectiveness of a porphyrin-based targeted radionuclide therapy has been demonstrated for potential use in the diagnosis and treatment of cancer. However, tumor treatment by radiation in combination with chemotherapy and EP can provide another effective line of therapeutic strategy. Recent research directed towards application of EP in cancer therapeutics has led to the emergence of ECT involving enhanced delivery of drugs to the cancer cells (Gothelf et al. 2003). Therefore, the present investigation was undertaken with the aim to study the effect of chemoradiotherapy in combination with EP using thymic lymphoma tumor model. In the present study, a Medical Electroporator (Model No. RB&HSD/01-04, BARC) developed in-house was used for application of electric pulses. A few initial in vitro experiments were conducted to establish the performance and efficacy of the electropulsator by using fibrosarcoma and Ehrlich's ascites carcinoma (EAC) cells. Application of EP (2.0 kV/cm) could kill 12.2% of the fibrosarcoma cell, but treatment with 3 Gy prior to EP could enhance the cells killing up to 18% (Figure 4.7). Exposure of EAC cells to 3 Gy and EP induced about 14 and 25% killing respectively; whereas the combination of both could remarkably increase (38.27%) the effect at 3h post-treatment (Figure 4.6).

The cytotoxic potential of radiation and the antineoplastic drug was demonstrated in the TL cells. The TL tumor resembled non-Hodgkin's lymphoma (NHL) (Chapter 2, Section 2.3.1.2) which in human is treated with a combination of drugs viz. cyclophosphamide, Dox hydrochloride and vincristine. Therefore further studies were undertaken to investigate the effect of the antineoplastic drugs in the inhibition of growth of the TL tumor. Cyclophosphamide at a dose of 5 mg when mixed with the tumor cells and transplanted to mice could completely inhibit the growth of the tumor up to 30 d (Table 4.3). Vinblastin at the maximum concentration (0.05 mg) inhibited tumor growth in 40% of the mice up to 40 d (Table 4.4). The tumor
growth inhibition with Dox (0.125 mg) treatment was 100% up to 60 d (Table 4.5). However, Dox administered i.v. at the dose of 0.125 mg to the mice bearing relatively smaller sized (0.004 cm³) tumor could inhibit the tumor growth in 40% of the animals. To get the complete tumor inhibition the dose of Dox was to increase by 2 fold (Table 4.6).

After observing the viability pattern (Figure 4.4) in electroporation medium at 37°C up to 3 h, the TL cells were examined for sensitivity to γ-irradiation and EP in vitro. Viability of the cells was found to decrease by 8.78 and 20.0% with irradiation (3 Gy) and EP (2.0 kV/cm) respectively at 3h post-treatment which was further reduced to 28.36% by irradiation prior to application of EP (Figure 4.10). Study carried out to measure the intracellular ROS generation showed dose dependent increase in the fluorescence intensity in the TL cells due to the treatments. The ROS generation was more intense (>2.5 fold) due to treatment with radiation in combination with EP (Figure 4.13).

The study revealed that the combination treatment was suitable to kill the tumor cells to a reasonable extent at relatively moderate dose of γ-irradiation and low electric field strength of EP. Moreover, attempt was made to study the cytotoxic effect of one of the common antineoplastic drugs, Dox in combination with EP. TL cells treated with Dox (5 and 10 µg) or EP showed marginal decrease (about 8 and 16 or 19%, respectively) in their viability. However, when Dox is added to the tumor cells before EP treatment the cytotoxicity enhanced drastically (Figure 4.14). The decrease in viability of the TL cells due to the treatment was also confirmed by measuring the apoptotic index. Increase in apoptotic index was proportional to the degree of cytotoxicity elicited by the treatment combination (Figure 4.15). Similar trend was also exhibited by the cells in terms of generation of intracellular ROS (Figure 4.16) indicating effectiveness of the treatments. Combination treatment of EP with Dox at concentrations 5 and 10 µg effectively decreased the tumor cell viability by 1.8 and 3.3 folds, respectively.
Treatment of TL cells with radiation (3 Gy) caused about 7.5 % decrease in the cell viability (Figures 4.8) and Dox (5 and 10 µg) caused about 8 and 16 % decrease in viability of the cells (Figures 4.14) at 3 h of incubation, which was not very significantly high. Therefore a study was designed to investigate the doses of radiation that might cause delay in appearance and growth of TL tumor. Appearance and growth of TL was unaffected by 2 and 4 Gy of irradiation. Irradiation of the transplanted tumor cell with 8, 12 and 16 Gy delayed the appearance of tumor in 20, 20 and 40% of the animals, respectively (Table 4.1). Similarly tumor growth inhibition of 7.7 and 13.6 % could be achieved by exposing the TL cells to 2 and 4 Gy, respectively on 12 d of transplantation (Table 4.2).

Therefore, the study was further extended to in vivo TL tumor model in Swiss mouse. Regression or inhibition of growth of TL tumor was studied in female Swiss mice following treatment with radiation, EP and Dox severally and in combination with a view to explore the possibility of developing an effective locoregional tumor treatment strategy. The growth kinetics of the tumor showed that the average tumor volume was increased by about 8 and 17 % following treatment with radiation (2 Gy) or Dox (10 µg), and decreased by about 17 % following treatment with EP (2kV/cm) compared to control on 7 d. The reduction in tumor volume however, was 47.88% with the combination therapy of radiation, Dox and EP (Figure 4.17).

The average DT of the tumor in the combination treatment group (1.18 ± 0.14 d) was comparable to that of control animals (1.03 ± 0.06 d) on 7 d. The average SGR of TL tumor was markedly decreased from 0.68 ± 0.04 %/d in control animals to 0.59 ± 0.08 %/d in the treated animals (Table 4.7). As compared to DT, SGR of tumor is considered more accurate (Mehrara et al. 2007) to use for growth fraction and growth rate heterogeneities within the tumor, although, some degree of uncertainty due to measurement technique and investigator may propagate to DT and SGR (Tong et al. 1998). However, the combination therapy is seen efficacious in this unitary study and warrant further exploration for its potential use, particularly, in those cancers where it is doubtful or difficult to perform surgical treatment.