**The response of human colorectal xenografts with abnormal Tp53
status to chronomodulated Topotecan and X-radiation treatments**

by

DALJIT K. DHALIWAL

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of

Master of Science

Ottawa-Carleton Institute for Physics
Department of Physics
Carleton University
Ottawa, Ontario
August, 2008

©2008, Daljit K Dhaliwal

Library and
Archives Canada

Bibliothèque et
Archives Canada

Published Heritage
Branch

Direction du
Patrimoine de l'édition

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

ISBN: 978-0-494-44121-3

Our file Notre référence

ISBN: 978-0-494-44121-3

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Abstract

Previous study with human colorectal cancer(HCT116) p53+/+ xenografts showed greatest treatment efficacy and least normal tissue toxicity in the late active period of mice. In this study, mice bearing p53-/- tumors were treated with both 6 and 12mg/kg Topotecan (TPT) or 16Gy of X-Radiation (XR) for 2 weeks. The Mann-Whitney, a non-parametric test, was used for the analysis. The results with 12mg/kg TPT were in general agreement with the previous study, with the active phase of mice (9pm, 3am) having least normal tissue toxicity as compared to the rest phase (9am, 3pm). Some differences were observed between 6 and 12mg/kg TPT, suggesting a possible concentration-related effect. The XR results suggested that the previously found optimal time of treatment (3am) applied as well to HCT116 tumors with p53-/- status. Overall, our results suggested potential for chronomodulating XR treatments, regardless of p53 status, indicating potentially more widespread application for this approach.

Acknowledgement

I thank my supervisor, Dr. Cheng Ng, for guiding this research and for his continuous encouragement and support. I am grateful to Denise Proulx for her immense help in this study. Her expertise and valuable suggestions have contributed a lot in the chronomodulation project. I would like to thank Bradley Scott for his support through out my research. I wish him luck with his future work. I would also like to thank Dr Gosia Niedbala for her guidance in the calibration of the irradiator. Her unique and insightful way of approaching the problems has taught me a lot. My heartfelt thank you to my friends Lourdes Garcia-Fernandez, Sorina Truica, Nadia Riaz (Wayne State University), Neetu Shukla (Oakland State University), and Perminder Cheema.

Finally, I am grateful to my parents for their encouragement and constant support. My special thank you to my sister Mandeep Benipal, who has motivated me a lot in the successful completion of this work. Last but not the least I thank my husband for his support through out my Master's degree.

Contents

Abstract	ii
Acknowledgement	iii
Contents	iv
List of Figures	vii
List of Tables	xi
List of Abbreviations	xv
List of Terms	xvi
1 Introduction	1
1.1 Circadian rhythms in normal and tumor tissues	2
1.2 Clinical data	3
1.3 Chronomodulated treatments	4
1.3.1 Chronomodulation of Topotecan	4
1.3.2 Chronomodulation of X-Radiation	5
1.4 Tumor suppressor p53	7

1.5	Previous results	8
1.6	Goals and hypothesis	9
2	Experiment setup	10
2.1	Materials and methods	12
2.1.1	Treatment	12
2.1.2	Effectiveness of treatment	13
2.1.3	Statistical analysis	15
2.2	Calibration of the irradiator	17
2.2.1	Half value layer (HVL)	17
2.2.2	Beam Flatness	21
2.2.3	Correction factors for dose rate	22
2.2.4	Backscatter factors	29
3	Results	36
3.1	Chronomodulation of Topotecan(12 mg/kg)	37
3.1.1	Tumor Volume	37
3.1.2	Body Weight	45
3.1.3	Leukocyte count	52
3.2	Chronomodulation of Topotecan(6 mg/kg) and X-Radiation (4 Gy/fraction)	58
3.2.1	Tumor volume	58
3.2.2	Body Weight	68
3.2.3	Leukocyte count	77
4	Analysis	84

4.1	Chronomodulation of TPT (12 mg/kg)	84
4.2	Chronomodulation of TPT (6 mg/kg) and XR (4 Gy/fraction) . .	86
4.2.1	Tumor volume	86
4.2.2	Body weight	88
4.2.3	Leukocyte count	90
5	Conclusion	92
5.1	Future studies	94
	Bibliography	96

List of Figures

1.1	Schematic representation of cell cycle phases	6
2.1	Percentage depth dose plotted against depth for the clinical irradiator with 1.65 mm Al filter and SSD 30 cm at 100 kVp	18
2.2	Graph of Half Value Layer (HVL) for Al filters of different known thicknesses (mm). The SSD was set at 30 cm for tube potential of 100 kVp	20
2.3	Plot of exposure vs optical density of different x-ray films. The SSD was set at 30 cm with tube potential of 100 kVp	23
2.4	Field size for the x-ray film radiated for 6 sec at the tube potential of 100 kVp	24
2.5	The in-plane profile of the x-ray beam for field size of 8 cm at 100 kVp	25
2.6	The cross-plane profile of the x-ray beam for field size of 8 cm at 100 kVp	26
2.7	Plot of water kerma based back scatter as a function of HVL (mm Al) for different field sizes [13]. Backscatter factors are the water-kerma ratio (i.e. unitless).	30

2.8	Plot of water kerma based back scatter factors (BSF) as a function of field size (cm) corresponding to the HVL of 2.63 mm Al	32
2.9	Plot of time (sec) vs the absorbed dose (cGy)	33
2.10	The plot of BSF ratio vs the diameter (cm). Error bars are too small to be visible.	35
3.1	Median tumor volume of mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO) compared with the matched control	41
3.2	Median tumor volume of mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO) compared with the matched control	42
3.3	Median tumor volume of mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO) compared with the matched control	43
3.4	Median tumor volume of mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO) compared with the matched control	44
3.5	Median body weight of mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO) compared with their matched control	48
3.6	Median body weight of mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO) compared with their matched control	49
3.7	Median body weight of mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO) compared with their matched control	50
3.8	Median body weight of mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO) compared with their matched control	51
3.9	Leukocyte count of mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO) compared with their matched control	54

3.10 Leukocyte count of mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO) compared with their matched control	55
3.11 Leukocyte count of mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO) compared with their matched control	56
3.12 Leukocyte count of mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO) compared with their matched control	57
3.13 Tumor volume of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 a.m. (3 HALO) compared with their matched control	64
3.14 Tumor volume of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 p.m. (9 HALO) compared with their matched control	65
3.15 Tumor volume of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 p.m. (15 HALO) compared with their matched control	66
3.16 Tumor volume of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 a.m. (21 HALO) compared with their matched control	67
3.17 Median body weight of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 a.m. (3 HALO) compared with their matched control	73
3.18 Median body weight of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 p.m. (9 HALO) compared with their matched control	74

3.19 Median body weight of mice treated with TPT (6 mg/kg) and XR (6 Gy/fraction) at 9 p.m. (15 HALO) compared with their matched control	75
3.20 Median body weight of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 a.m. (21 HALO) compared with their matched control	76
3.21 Leukocyte count of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 a.m. (3 HALO) compared with their matched control	80
3.22 Leukocyte count of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 p.m. (9 HALO) compared with their matched control	81
3.23 Leukocyte count of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 p.m. (15 HALO) compared with their matched control	82
3.24 Leukocyte count of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 a.m. (21 HALO) compared with their matched control	83

List of Tables

2.1	Half value layer (HVL) data taken for different Al filters at source surface distance (SSD) of 30 cm and tube potential 100 kVp . . .	19
2.2	Optical density readings of x-ray films for different exposure times at the tube potential of 100 kVp	21
2.3	The ionization chamber readings for V_H and V_L to determine the correction factor for ion collection efficiency	28
2.4	The ionization chamber readings for M_{raw}^+ and M_{raw}^- to determine the correction factor for polarity effects	28
2.5	The back scatter values corresponding to the HVL of 2.63 mm Al, interpolated from the data taken from the AAPM protocol [13] .	29
2.6	The corrected electrometer readings for different radiation times .	31
2.7	Back scatter factors interpolated for 2.63 mm Al and 8 cm diameter	34
2.8	The irradiation time for different cutout shields	34
3.1	Summary of the relationship between the phases (active or rest) and four different treatment times followed in the experiment . . .	37
3.2	The statistical analysis results of tumor volume for mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO)	39

3.3	The statistical analysis results of tumor volume for mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO)	39
3.4	The statistical analysis results of tumor volume for mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO)	40
3.5	The statistical analysis results of tumor volume for mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO)	40
3.6	The statistical analysis results of body weight for mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO)	45
3.7	The statistical analysis results of body weight for mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO)	46
3.8	The statistical analysis results of body weight for mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO)	46
3.9	The statistical analysis results of body weight for mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO)	47
3.10	Results of the statistical analysis of leukocyte count for mice treated TPT (12 mg/kg) at 9 a.m. (3 HALO)	52
3.11	Results of the statistical analysis results of leukocyte count for mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO)	53
3.12	Results of the statistical analysis of leukocyte count for mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO)	53
3.13	Results of the statistical analysis of leukocyte count for mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO)	53
3.14	The statistical analysis results of tumor volume for mice treated with TPT (6mg/kg) at 9 a.m. (3 HALO)	59

3.15	The statistical analysis results of tumor volume for mice treated with TPT (6mg/kg) at 3 p.m. (9 HALO)	60
3.16	The statistical analysis results of tumor volume for mice treated with TPT (6 mg/kg) at 9 p.m. (15 HALO)	60
3.17	The statistical analysis results of tumor volume for mice treated with TPT (6 mg/kg) at 3 a.m. (21 HALO)	61
3.18	The statistical analysis results of tumor volume for mice treated with XR (4Gy/fraction)at 9 a.m. (3 HALO)	61
3.19	The statistical analysis results of tumor volume for mice treated with XR (4Gy/fraction) at 3 p.m. (9 HALO)	62
3.20	The statistical analysis results of tumor volume for mice treated with XR (4Gy/fraction) at 9 p.m. (15 HALO)	62
3.21	The statistical analysis results of tumor volume for mice treated with XR (4Gy/fraction) at 3 a.m. (21 HALO)	63
3.22	The statistical analysis results of body weight for mice treated with TPT (6 mg/kg) at 9 a.m. (3 HALO)	69
3.23	The statistical analysis results of body weight for mice treated with TPT (6 mg/kg) at 3 p.m. (9 HALO)	69
3.24	The statistical analysis results of body weight for mice treated at 9 p.m. (15 HALO) with TPT (6 mg/kg)	70
3.25	The statistical analysis results of body weight for mice treated at 3 a.m. (21 HALO) with TPT (6 mg/kg)	70
3.26	The statistical analysis results of body weight for mice treated at 9 a.m. (3 HALO) with XR (4 Gy/fraction)	71

3.27	The statistical analysis results of body weight for mice treated at 3 p.m. (9 HALO) with XR (4 Gy/fraction)	71
3.28	The statistical analysis results of body weight for mice treated at 9 p.m. (15 HALO) with XR (4 Gy/fraction)	72
3.29	The statistical analysis results of body weight for mice treated at 3 a.m. (21 HALO) with XR (4 Gy/fraction)	72
3.30	The statistical analysis results of leukocyte count for mice treated with TPT (6 mg/kg) at 9 a.m. (3 HALO)	78
3.31	The statistical analysis results of leukocyte count for mice treated with TPT (6 mg/kg) at 3 p.m. (9 HALO)	78
3.32	The statistical analysis results of leukocyte count for mice treated with TPT (6 mg/kg) at 9 p.m. (15 HALO)	78
3.33	The statistical analysis results of leukocyte count for mice treated with TPT (6 mg/kg) at 3 a.m. (21 HALO)	78
3.34	The statistical analysis results of leukocyte count for mice treated with XR (4 Gy/fraction) at 9 a.m. (3 HALO)	79
3.35	The statistical analysis results of leukocyte count for mice treated with XR (4 Gy/fraction) at 3 p.m. (9 HALO)	79
3.36	The statistical analysis results of leukocyte count for mice treated with XR (4 Gy/fraction) at 9 p.m. (15 HALO)	79
3.37	The statistical analysis results of leukocyte count for mice treated with XR (4 Gy/fraction) at 3 a.m. (21 HALO)	79

List of Abbreviations

ALL	Acute Lymphoblastic Leukemia
BSF	Back Scatter Factors
CPT	Camptothecin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
HALO	Hours After Lights On
H-D curves	Hurter and Driffield Curves
HVL	Half Value Layer
KVp	Peak Kilovoltage
PBS	Phosphate Buffered Saline
PDD	Percentage Depth Dose
SCLC	Small Cell Lung Cancer
SCN	SupraChiasmatic Nucleus
SSD	Source Surface Distance
Topo	Topoisomerase
TPT	Topotecan
XR	X-Radiation

List of Terms

Absorbed Dose:

Absorbed dose, also known as total ionizing dose (TID), is the amount of energy deposited by the ionizing radiation in the medium. It is measured in gray (Gy), where 1 Gy is the energy deposited per unit mass of the medium i.e. J/kg.

Actigraph:

It is a non-invasive device worn on the wrist to monitor human rest/activity cycles.

Apoptosis:

Apoptosis, also referred to as cell suicide, is a form of programmed cell death in which series of molecular steps lead to cell death. In cancer cells this process may be hindered.

Attenuation Coefficient (μ):

It is a term used to describe the extent to which the intensity of the energy beam decreases after passing through a specific material. It is measured in cm^{-1} .

Camptothecin (CPT):

Camptothecin is a cytotoxin anti-cancer drug that inhibits DNA topoisomerase enzyme (topo I).

Camptothecin Drugs:

Anticancer drugs which are related to camptothecin in structure, and inhibit the DNA enzyme topoisomerase.

Chronomodulation:

It is the delivery of the treatment at different times of day and night.

Circadian rhythm:

Circadian rhythms are approximately 24h cycles that regulate many physiological, biochemical, and behavioral processes in all higher forms of life.

DNA:

Deoxyribonucleic acid is a linear, unbranched nucleotide that codes genetic information for transmission of inherited traits.

Dosimeter:

It is a device used to measure exposure to a hazardous environment.

Fractionated X-Radiation:

If XR is fractionated with one or more rest periods in between, fewer cells die due to their self repair mechanisms which repair damaged DNA and other biomolecules.

Grayscale:

It is the value of each pixel that carries complete information about the intensity of the radiation.

HALO:

HALO, an acronym for Hours After Lights On, is a term used for experimental rodents that allows easy comparison of studies in different labs without depending on their light/dark cycles.

H and D curve:

H and D curves, also known as characteristic curves, give the relation between film density and log of exposure in mR.

Half Value Layer (HVL):

Half value layer is the thickness of the absorbing material (Al or Cu) required to reduce the air-kerma rate by 50% in the narrow beam conditions.

Hypoxia (Oxygen Effect):

It is a condition wherein the oxygen supply to the tissue decreases. The level of hypoxia in a tumor may help predict the tumor response to cancer treatment.

Intra-peritoneal:

Abbreviated as IP, it is a term used when the drug is administered through the peritoneum, a thin and transparent membrane lining the wall of abdomen.

Kerma:

Kerma, an acronym for Kinetic Energy Released per unit Mass, is the sum of kinetic energies of all charged particles liberated by indirectly ionizing particles like photons and neutrons.

LD₅₀:

Lethal dose or LD₅₀ is the amount of a material which if given all at once, can cause the death of 50% of a group of test animals. It measures the short term toxicity of a material.

Leukocyte:

White blood cells, or leukocytes, are the cells of the immune system made by bone marrow. These include lymphocytes, neutrophils, eosinophils, macrophages, and mast cells.

Melatonin:

It is a hormone secreted by the pineal gland that helps control the body's sleep cycle, and is also an anti-oxidant.

Optical Density:

The degree to which an x-ray film is blackened is related to the intensity of the radiation reaching the film. The amount of blackness on the film is called optical density.

P53:

P53, also referred to as protein 53 or tumor protein 53, is located on the short arm of chromosome 17 and functions as tumor suppressor.

Pharmacokinetics:

It is a term used to define the activity of drugs in the body over a period of time i.e. how the drugs are metabolized, delivered, localized and excreted.

Topotecan (TPT):

It is a CPT analog used to treat ovarian cancer, small cell lung cancer, and cervical cancer. It is sold under the commercial name Hycamtin by SmithKline Beecham.

Transcription of DNA:

It is process by which a cell synthesis mRNA from a DNA template. It is like replication of DNA in that a DNA strand is utilized to synthesize a strand of mRNA.

Translation of DNA:

It is a process by which ribosomes synthesize proteins using mRNA.

Xenograft:

It is a surgical graft of tissue from one species to another species.

Chapter 1

Introduction

Circadian rhythms, which are approximately 24h cycles, regulate many physiological, biochemical and behavioral processes in all higher forms of life. These rhythms have genetic basis and are driven by an internal clock.

In mammals, these circadian rhythms have been attributed to the complex molecular loops involving at least nine specific genes known so far. These are: period(per) 1, per 2, per 3, Cryptochrome(cry) 1, cry 2, Timeless(tim), clock, bmal1, and Casein kinase 1 (CKI). The suprachiasmatic nucleus (SCN), located on the hypothalamus, is the core component of the central clock. The alternation between the states of high activity and recuperation are coordinated by the SCN. The visual pathways and melatonin, a hormone secreted by the pineal gland during darkness, synchronize the SCN by acting as inputs of environmental conditions [1].

The synchronized 24h rhythms are observed at various cellular and molecular levels such as metabolism, cell proliferation, cell death, transcription and translational processes, blood flow, among many other important biological functions.

1.1 Circadian rhythms in normal and tumor tissues

Studies have indicated that the circadian rhythms in the cellular metabolism and proliferation activity of tumors exist with different amplitude and cell cycle phase compared to normal tissues [3] [1]. Chronotherapy of cancer exploits these differences in normal and tumor tissues for therapeutic gain. These variations appear to regulate the amount of metabolism of intracellular cytotoxic substances leading to cell death, damage or cell repair [2]. Thus, the concept of circadian treatment delivery is based on the need to minimize the damage to normal tissue while maximizing the damage to tumor cells. This depends on factors such as amplitude and cell cycle phase susceptibility of normal or tumor tissues. The increase in host tolerance of the cytotoxic treatment agent at specific circadian times provides an opportunity to safely escalate the dose so as to maximize the killing of tumor cells [3].

Actively proliferating cells have been known to be very sensitive to the treatment agent. These include cells in bone marrow, gastrointestinal stem cells, epidermal stem cells, spermatogonia and erythroblasts. The body's most sensitive cells are the undifferentiated, well nourished, and rapidly proliferating cells with high metabolic activity. Their high sensitivity limits the dose of anticancer agents.

The biological functions like blood flow, circulating blood cells, plasma protein, liver metabolism, etc also affect the sensitivity of the normal or tumor tissue to the treatment agent. The reasons could be the proportion of cells in a particular cell cycle phase which might be sensitive or resistant to the treatment agent at the time of exposure, tumor blood flow affecting the amount of drug delivered

and oxygenation of the targeted tissue, or the rhythmicity in the activity of the enzyme that metabolizes the drug. The pharmacokinetics of many anticancer agents have been studied on laboratory rodents as well as cancer patients [2].

1.2 Clinical data

The clinical trials testing the circadian dosing time have suggested significant clinical benefits from chemotherapy or radiotherapy. Circadian scheduling of doxorubicin and cisplatin in patients with widespread ovarian cancer allowed safe administration of higher doses along with a four-fold 5 year survival advantage [4]. The absolute cure frequency of children with acute lymphoblastic leukemia [ALL] increased by almost 20% when treated at optimal evening timing with 6-mercaptopurine (6MP) and methotrexate [4][5]. The chronomodulated infusion of 5-Fluorouracil (5-FU) during the late activity (sleep span) for colorectal cancer patients has demonstrated several-fold increase [4][6]. Randomized clinical trials in patients with metastatic colorectal cancer have shown two-fold increase in the objective response frequency after chronomodulated treatment with 5-FU/leukovorin(LV) and oxaliplatin [4][6].

The circadian variations in cell cycle related parameters studied in 30 ovarian cancer patients showed almost 12h phase difference in the DNA synthesis of normal and tumor cells. In patients with non-Hodgkin's lymphoma, circadian maximum of DNA synthesis occurred near midnight while the S-phase (synthesis phase) peaked in the bone marrow of healthy subjects between 12:00 hr and 16:00 hr [7]. In another study of 33 patients with advanced ovarian or metastatic breast cancer, circadian rhythms were observed in the cortisol, circulating blood cell counts

and plasma proteins. These clinical trials of chronomodulated treatments and circadian patterns in cell cycle related parameters have provided evidence of significant therapeutic gain. The non-invasive actigraph device to detect circadian rhythms and the availability of programmable infusion pumps have strengthened the potential of chronotherapeutic regimen.

1.3 Chronomodulated treatments

In this study, a potential therapeutic gain was studied for the chronomodulated treatments with Topotecan (TPT) or X-radiation(XR).

1.3.1 Chronomodulation of Topotecan

Topotecan is a chemotherapy drug that belongs to the class of camptothecin (CPT) analogues like irinotecan (CPT-11), and 9-aminocamptothecin (9-AC). CPT analogues are cytotoxic anticancer drugs that inhibit DNA topoisomerase I (topo I). DNA topo I is an enzyme that regulates DNA supercoiling which is crucial during DNA transcription and replication. As topo I generates DNA strand breaks, they are targeted by small molecule inhibitors like CPT-11, TPT, and 9-AC. Irinotecan is most commonly used for treating colon cancer, in particular as a combination with other chemotherapy agents like 5-FU, and leucovorin (LV). Topotecan, the first inhibitor for oral use, is used to treat ovarian, small cell lung cancer (SCLC) and advanced cervical cancer. The CPT analogues target S-phase cells.

Because of the difference in the DNA synthesis of normal and tumor tissues, the chronomodulation of topotecan could potentially prove to increase the therapeu-

tic benefits.

1.3.2 Chronomodulation of X-Radiation

Radiation therapy usually involves treating cancer with gamma rays, x-rays etc. The primary target for using radiation is to destroy the genetic structure of the cell and inhibit its ability to divide and grow. It is known that the rapidly dividing cells are more sensitive to X-radiation than the non proliferating cells. Tumor cells that are actively proliferating are more susceptible to radiation damage than the normal cells.

The sensitivity of cells to x-radiation in all cell cycle phases is different. Cells in the mitotic phase are known to be the most sensitive cells. In gap G2 also, their sensitivity is somewhat similar to the mitotic phase. The G1 phase, which is of longest duration out of all cell cycle phases, is somewhat radio-resistant in the early phase and sensitive towards the end. The S-phase (synthesis phase) of the cell cycle is most resistant to radiation. The presence of two identical DNA double helix molecules are thought to initiate DNA repair following the completion of replication in the S-phase [8]. Figure 1.1 shows the different cell cycle phases. The rapidly dividing cells in bone marrow, crypt-epithelium of the intestine, endothelial cells etc limit the radiation dose that can be delivered.

It has been shown that LD_{50} for mice given whole body radiation at six circadian times varied between 400-500R. The second half of the rest phase showed highest tolerance. The shortest survival time was found during late dark (active) or early light span [9]. Not much clinical data is available for circadian timed fractionated X-radiation treatments.

The difference in phase and amplitude of normal and tumor cells could be utilized

Figure 1.1: Schematic representation of cell cycle phases. (Source: <http://herb4cancer.files.wordpress.com>)

to chronomodulate X-radiation to increase the therapeutic window.

1.4 Tumor suppressor p53

p53 (protein 53) encoded by the TP53 (tumor protein 53) gene, located on the short arm of chromosome 17, is an important tumor suppressor. p53 is normal in only about 50% of human tumors, though speculated to be dysfunctional through disturbed regulation or protein-protein interaction. In the tumors with TP53 mutations, protein functions are totally impaired.

Activated by several factors like DNA damage, hypoxia, nucleotide imbalance, spindle damage or oxidative stress, p53 can halt cell cycle progression; direct DNA damage repair or induce apoptosis (programmed cell death). p53 functions by acting as a transcription factor, though it is not clear how the decision of cell cycle arrest or apoptosis is made. Cell cycle arrest is usually made in the G1 and G2 phases in response to DNA damage. It repairs the damaged DNA by regulating the transcription and interacting with the repair and recombination elements. If the DNA damage proves to be irreparable, it initiates apoptosis [10]. When both the alleles of p53 gene are mutated, it results in abnormal p53 (p53-/-) which then acts as an oncogene, leading to the tumor formation. Because of the mutations, it fails to sense the DNA damage and halt the cell cycle progression or induce apoptosis.

1.5 Previous results

The previous study [14] was done on mice bearing human colorectal HCT116, p53+/+ xenografts. The mice were treated for two weeks with either 12 mg/kg Topotecan (TPT) or X-radiation (XR) of 4 Gy/fraction alone during their active (i.e. dark phase) and rest phase (i.e. light phase). Mice are nocturnal, their active and rest phases are reversed from humans.

The four different times representing the active and rest phase were 9 a.m., 3 p.m., 9 p.m., and 3 a.m. Tumor volume was calculated to determine the efficacy of the treatment. Body weight and the number of leukocyte counts was used to evaluate the normal tissue toxicity. Mann-Whitney U test was used to do the statistical analysis.

It was concluded that the treatment time significantly affected the tumor efficacy and normal tissue toxicity. The treatment (TPT or XR) delivered during the late active period (3 a.m.) of mice was found to be most effective since it had greatest tumor efficacy along with significantly decreased normal tissue toxicity. However, the treatments delivered during the late rest period (3 p.m.) of mice were found to be least effective since the tumor efficacy was lowest among the tested groups and the normal tissue toxicity was highest. The tumor efficacy was assessed by the ability of the treatment agent (TPT or XR) to decrease the tumor volume. The normal tissue toxicity was assessed from decrease in the body weight and the peripheral blood leukocyte count.

1.6 Goals and hypothesis

Since p53 mutations exist in about 50% of human tumors, it is important to determine whether the previous results of enhanced therapeutic gain, associated with the TPT or X-radiation treatments, with normal p53+/+ human colorectal tumor(HCT) cells can be replicated in the abnormal HCT116 p53-/- cell line and, therefore, potentially to all human tumors regardless of the p53 status. The goal of this research was to determine the efficacy and normal tissue toxicity associated with TPT or fractionated X-Radiation treatments delivered to mice bearing HCT116 p53-/- xenografts at four different time points over day/night cycle.

The hypotheses to be tested were:

- The TPT or X-Radiation treatments delivered at 3 a.m. (late active phase) to mice bearing HCT116 p53-/- cell line will be associated with significantly enhanced treatment efficacy and decreased normal tissue toxicity, thus increasing the therapeutic gain.
- Since HCT116, p53-/- cell line is isogenic with HCT116, p53+/+ and differs only in the p53 status, the previous results of potentially enhanced therapeutic gain with HCT116, p53+/+ xenograft also extend to HCT116, p53-/- xenograft.

Chapter 2

Experiment setup

For the chronomodulation study, CD-1 nude female mice (Charles river laboratories, Wilmington, MA) were used as for the previous work [14]. Since the nude mice are immune compromised, they were kept in autoclaved housing at the University of Ottawa's animal care facility and were given sterilized food and acidic water. The measurements for tumor volume and body weight were done in a hood while wearing sterile gown and gloves, face mask, hair net and shoe covers. The mice were eight weeks at the time of tumor injections. The experiment protocol was approved by University of Ottawa's animal care facility. All efforts were done to provide animal care in accordance with their ethical guidelines.

The treatment was given with either TPT or X-radiation at four different times spread over 24h cycle of day and night. The four different treatment times for the experiment were: 9 a.m.(3 HALO), 3 p.m.(9 HALO), 9 p.m.(15 HALO), and 3 a.m.(21 HALO). The term HALO (Hours After Light Onset) allows easy comparison of studies done in different labs without depending on their light/dark cycles [11]. To achieve the effect of treatment at night, half of the mice were light

shifted by 12h. For the mice in normal light cycle, lights were on at 6 a.m. and off at 6 p.m. while the mice in the reversed group had lights on at 6 p.m. and off at 6 a.m. The day and night cycles of mice are opposite to humans. The change from normal to reverse light cycle was brought gradually over the first four days. On day 1, lights were off at 3 p.m. and on at 3 a.m.; on day 2, lights were off at 12 noon and on at 12 midnight; on day 3, lights were off at 9 a.m. and on at 9 p.m.; and on day 4, lights were off at 6 a.m. and on at 6 p.m..

The light shifted mice were placed in the shifted cycle for two weeks before the start of experiment to allow for adjustment of chronobiological rhythms. For this reason, the reverse light cycle mice were two weeks younger i.e. six weeks at the time of shifting the cycle. This segregation allowed us to administer treatments and perform other measurements during the day. The time shifting protocol was similar to that used previously [14].

2.1 Materials and methods

The tumors were established using HCT116 p53^{-/-} cell-line. The HCT116 (Human Colorectal Tumor cells) were obtained from Dr. B Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). Cultured tumor cells were suspended in serum free Dulbecco's Modified Eagle's Medium (DMEM) at a concentration of 2.5×10^7 cells/ml. For formation of adequate tumor size, 200 μ l of this solution was injected sub-cutaneously in each mouse and allowed to grow for 12 days before administering first treatment.

2.1.1 Treatment

The treatment was done with either Topotecan (TPT) or X-radiation alone. For each treated group, we had the corresponding control mice for all time points. The control mice underwent identical conditions of stress and handling as the treated mice. These mice were sham injected with phosphate buffered saline (PBS) solution on the days of treatment.

Topotecan

The drug Hycamtin (common name: Topotecan) obtained from SmithKline Beecham, Oakville, ON was used in the study. 4 mg of active drug (topotecan hydrochloride) was present in the 72 mg vial of Hycamtin that also contained 48 mg mannitol and 20 mg tartaric acid as its inactive ingredients. In line with the study done with HCT116 p53^{+/+} cell-line, initial injection dose was fixed at 12 mg/kg body weight. A solution at concentration 3 mg/ml of active drug dissolved in PBS was used for the intra-peritoneal injections. The mice were in-

jected four times over a period of two weeks i.e. on day 0, 4, 7 and 10 where day 0 was considered as the first day of the treatment. However, 12 mg/kg dose was found to be high for this study. So, a lower dose of 6 mg/kg was used after initial experiments and was found sufficient to give conclusive results. A solution of 1.5 mg/ml of active drug was dissolved in PBS for this dose.

X-radiation

The X-radiation was given using the research irradiator, Pantek Bipolar Model HF320, at the Ottawa hospital, General campus. As in the previous experiment with HCT116 p53+/+, a dose of 4 Gy was given four times over a period of two weeks i.e. on day 0, 4, 7 and 10 [14]. Hence, a total dose of 16 Gy was delivered to the tumor of each mouse. The dose to the tumors was calculated in accordance to the in-air calibration method as described in the AAPM protocol [13].

2.1.2 Effectiveness of treatment

The efficacy of the treatment was determined by the tumor regrowth assay while the decrease in the body weight and the number of leukocyte counts indicated toxic effects to the normal tissue.

Treatment efficacy

To determine the tumor regrowth, tumor volumes were calculated for a period of five weeks starting from the first day of treatment. Assuming the tumors to have ellipsoidal shape, the tumor volume was calculated using the formula:

$$V = \frac{\pi}{6} \times l \times w \times d \quad (2.1)$$

derived from the volume of sphere,

$$V = \frac{4}{3} \times \pi \times r^3 \quad (2.2)$$

where $l = w = d = 2r$. l , w and d were the length, width and depth of the tumor measured with sterilized vernier callipers [14]. The institutional guidelines at the animal care facility recommended an endpoint for the mouse if any dimension exceeded 16 mm or if ulceration was observed in the tumor.

Treatment toxicity

To determine the toxic effects of the treatment agent on the normal tissue, two different parameters were observed: body weight and peripheral blood leukocyte count. The body weight measurements were done twice a week for five weeks, starting from the first day of treatment [14]. For measuring changes in the leukocyte count, blood samples were taken from tail vein of mice once a week for five weeks. Bright line haemocytometer was used to count the number of leukocytes [14].

2.1.3 Statistical analysis

The number of mice left alive at the end of experiment was different for different time points of all groups: TPT or X-radiation treated and control group. During the experiment, a few mice were lost due to various reasons, e.g. ulceration, any tumor dimension exceeding 16 mm, and weight loss according to the guidelines of the animal care. Therefore, Mann-Whitney U test, which is a non-parametric test to compare two independent groups, was used to do the analysis. A non-parametric test makes no assumption about the distribution of data i.e. whether it is gaussian or not. This test compares sum of ranks and assumes that the data was taken from two independent random samples that have same shape [12].

To apply Mann-Whitney test, data from two samples: sample A and B are combined and ranked from lowest to highest. If N is the total sample size; n_A and n_B are the number of observations in samples A and B with rank sum of R_A and R_B respectively, then:

$$N = n_A + n_B \quad (2.3)$$

Ranks of observations in the two samples are added and value for U is calculated:

$$R_A + R_B = \frac{N(N+1)}{2} \quad (2.4)$$

$$U = n_A n_B + \frac{n_B(n_B+1)}{2} - \sum_{i=n_A+1}^{n_B} R_i \quad (2.5)$$

where R_i is the rank. The probability or the p-value for calculated U was obtained using GraphPad prism software (GraphPad software, Inc., San Diego, California, USA). The significance level of 5% (pre-chosen probability) was used to indicate considerable differences between two groups [14]. Hence, for $p < 0.05$, samples

were considered significantly different. The Mann-Whitney test however requires a total sample size of atleast 7 or more. For sample size of less than 7, p value was always greater than 0.05.

2.2 Calibration of the irradiator

Following the AAPM protocol [13], the in-air calibration method was used to calibrate the irradiator for low energy x-rays (100 kVp). The reference dosimetry was performed free in air and backscatter factors were included to account for the effect of the phantom scatter. The calibration included determining half value layer (HVL), measuring optical density to draw H-D curves, determining the beam flatness, and calculating the dose rate. The dose rate included correction factors like p_{pol} , p_{ion} , p_{TP} , back-scatter factors etc.

2.2.1 Half value layer (HVL)

Half value layer is the thickness of an absorbing material (Al or Cu) required to reduce the air-kerma rate by 50 percent in the narrow beam conditions [13]. A percentage depth dose (PDD) curve gives variation of relative dose with depth in the phantom (or patient). Figure 2.1 shows the PDD curve for an 8 cm cone with 1.65 mm Al filter and peak kilovoltage of 100 kVp [14][17]. At this tube potential, the dose delivered to the sensitive normal tissues (about 4 cm below the tumor), reduces to 35 % rendering it acceptable for radiating mice [14]. The HVL of research irradiator was determined by using Al filters of different thicknesses. The Al filter of thickness 2.27 mm was always used. The same ionization chamber (Model NE 2571 - Serial number 3465) was used along with the electrometer (Model NE 2570/1, Serial number 1358, a Farmer dosimeter from NE technology) to take the data for calibration. The source surface distance (SSD) was set at 30 cm. The ion chamber was adjusted at this level and exposure given for 1 minute, with tube parameters 12.5 mA and 100 kVp. The electrometer

Figure 2.1: Percentage depth dose plotted against depth for the clinical irradiator with 1.65 mm Al filter and SSD 30 cm at 100 kVp. (Source: Dana Mullins, "Chronomodulation of Topotecan and X-radiation therapy", Thesis, Carleton University, 2004; data taken from MacPherson, 1999.)

Table 2.1: Half value layer (HVL) data taken for different Al filters at source surface distance (SSD) of 30 cm and tube potential 100 kVp

Al Thickness(mm)	Reading 1	Reading 2	Reading 3	Average	Ratio
0.00	0.3250	0.3220	0.3220	0.3230	1.000
0.48	0.2720	0.2720	0.2720	0.2720	0.8421
0.96	0.2350	0.2350	0.2350	0.2350	0.7276
1.54	0.2100	0.2120	0.2100	0.2107	0.6522
2.02	0.1870	0.1870	0.1870	0.1870	0.5789
2.50	0.1670	0.1670	0.1670	0.1670	0.5170
3.10	0.1550	0.1550	0.1550	0.1550	0.4799

reading was taken three times for each filter thickness. To ensure no radiation was missed, the electrometer was started before starting the x-ray machine. In addition to the 2.27 mm Al filter, a lead aperture of diameter 2 cm and thickness 2 mm was also used. This ensured a field size of about 8 cm in diameter at the surface of tumor. Table 2.1 shows the electrometer reading for different Al filters used. The graph for this data is shown in fig 2.2.

The HVL is given by:

$$N = N_0 e^{-\mu x} \quad (2.6)$$

$$HVL = \frac{\ln(2)}{\mu} \quad (2.7)$$

where μ is the attenuation coefficient calculated from fig 2.2. The half value layer for the research irradiator was calculated to be 2.63 mm Al. This was compared with the clinical irradiator which had an HVL of 2.57 mm Al [14]. The percentage difference between the two values was estimated to be 2.33 %.

Figure 2.2: Graph of Half Value Layer (HVL) for Al filters of different known thicknesses (mm). The SSD was set at 30 cm for tube potential of 100 kVp. The error bars are too small to be visible. R^2 is the square of correlation coefficient and is the measure of reliability between X and Y values.

Table 2.2: Optical density readings of x-ray films for different exposure times at the tube potential of 100 kVp

Exposure time (sec)	Fog	Reading	Optical Density
2	0.1600	0.17	0.0100
4	0.1675	1.31	1.1425
5	0.1775	1.95	1.7725
6	0.1725	2.44	2.2675
7	0.1875	2.67	2.4825
8	0.1900	2.99	2.8000
9	0.1900	3.30	3.1100
10	0.1875	3.39	3.2025
20	0.2200	4.24	4.0200
30	0.2675	4.58	4.3125
40	0.3125	4.70	4.3875
50	0.3325	4.75	4.4175

2.2.2 Beam Flatness

The characteristic curves (also known as H and D curves after Hurter and Driffeld who explained them in 1890) give the relation between film density and log of exposure in mR [15]. To check for beam flatness at SSD 30 cm, the H-D curves were plotted to find the linear region. X-ray film was placed on the top of solid water (approximately, 5 cm in thickness) to reach SSD. Another solid water sheet of thickness about 0.2 mm was placed on top of the film for build up. Different films were used for different exposure times. The films were given exposure for time, $t = 2, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40$, and 50 sec respectively. Only Al filter (2.27 mm) and Pb aperture were used and tube parameters were set at 12.5 mA and 100 kVp. The optical density measurements of the films were done using densitometer (Model TBX, serial number - 5418, Tobias associates Inc., Ivyland, PA) at the Ottawa civic hospital campus. Table 2.2 shows the optical density readings for different exposure times.

The plot of exposure versus optical density is shown in fig 2.3:

The x-ray film exposed for 6 sec was selected to check for field size and beam flatness, since it was in the linear range of the H-D curve. Fig 2.4 shows the field size for the film radiated for 6 sec at tube potential 100 kVp and SSD 30 cm.

The beam profile of this film was taken using Vider scanner to determine if the profile was flat. Fig 2.5 shows the in-plane beam profile i.e. in the direction perpendicular to the x-ray tube. Fig 2.6 shows the cross-plane profile i.e. in the direction from anode to cathode. Since the radiation dose dropped to half towards the edges of the 8cm diameter, care was specially taken during the treatment of mice to keep the tumor within 6cm diameter.

2.2.3 Correction factors for dose rate

Since the tumors were located on the backs of mice, the reference depth for determining absorbed dose was considered at the phantom surface ($Z_{ref} = 0$). The absorbed dose to water at this reference depth for the field size of approximately 8 cm $\times$ 8 cm is given by [13]:

$$D_{w,z=0} = MN_k B_w P_{stem,air} [(\frac{\mu_{en}}{\rho})_{air}^w]_{air} \quad (2.8)$$

where, M is the free-in-air chamber reading taken by placing the ionization chamber at $Z_{ref} = 0$ corrected for factors like temperature, pressure, ion recombination, effect of polarity, electrometer accuracy etc. N_k is the calibration factor of air kerma, B_w takes into account the backscattering from the phantom surface, $P_{stem,air}$ is the chamber stem correction factor taken as 1 here because for the given beam quality, same field size was used for the experiment. $[(\frac{\mu_{en}}{\rho})_{air}^w]_{air}$ is

Figure 2.3: Plot of exposure Vs optical density of different x-ray films. The SSD was set at 30 cm with tube potential of 100 kVp. The error bars are too small to be clearly visible. It is an empirical fit obtained using GraphPad prism software.

Figure 2.4: Field size for the x-ray film radiated for 6 sec at the tube potential of 100 kVp

Figure 2.5: The in-plane profile of the x-ray beam for field size of 8 cm at 100 kVp

Figure 2.6: The cross-plane profile of the x-ray beam for field size of 8 cm at 100 kVp

the mean mass energy absorption coefficient ratio for water to air averaged over the incident photon spectrum.

To account for the variation of mass with temperature and pressure, correction for the charge collected in the chamber was made with respect to the reference temperature and pressure. The correction factor is given by [13]:

$$P_{TP} = \frac{P_{ref}[kPa]}{P[kPa]} \frac{(T[^\circ C] + 273.2)}{(T_{ref}[^\circ C] + 273.2)} \quad (2.9)$$

With $T_{ref} = 22^\circ C$, $T = 19^\circ C$, $P_{ref} = 101.33$ kPa (760 mm of Hg) and $P = 101.45$ kPa (760.87 mm of Hg), P_{TP} was calculated to be 0.9887.

Since electrometer is used in conjunction with the ionization chamber to take the readings, they have to be calibrated. The ionization chamber (Model NE 2571) and the electrometer (Model NE 2570/1) were already calibrated and used as one instrument. The electrometer correction factor, P_{elec} was unity.

The absorbed dose is accurately determined if all the ions formed by radiation in the ionization chamber are collected. Sometimes, the ions of opposite charge recombine on their way to the collection electrode and are not recorded. The correction for ion collection efficiency is thus required. P_{ion} is determined by estimating the number of ions formed at two different voltages: normal collecting voltage (V_H) and half of the normal collecting voltage (V_L). P_{ion} is calculated as [13]:

$$P_{ion}(V_H) = \frac{1 - \frac{V_H^2}{V_L^2}}{\frac{M_{raw}^H}{M_{raw}^L} - \frac{V_H^2}{V_L^2}} \quad (2.10)$$

Table 2.3: The ionization chamber readings for V_H and V_L to determine the correction factor for ion collection efficiency

V_H	M_{raw}^H	V_L	M_{raw}^L
263.50	1.3150	137.50	1.3200
	1.3170		1.3150
	1.3150		1.3170
	1.3200		1.3170
average	1.3168		1.3173

Table 2.4: The ionization chamber readings for M_{raw}^+ and M_{raw}^- to determine the correction factor for polarity effects

Polarity V (-)	M_{raw}^-	Polarity V (+)	M_{raw}^+
263.50	-1.3150	268.00	1.3270
	-1.3170		1.3220
	-1.3150		1.3200
average	1.3157		1.3230

M_{raw}^H and M_{raw}^L are the raw chamber readings at voltage V_H and V_L respectively.

Table 2.3 shows the observed values of V_H , V_L , M_{raw}^H , and M_{raw}^L .

The P_{ion} was calculated to be 0.9999.

Correction is also required to account for the polarity effects due to beam quality and cable arrangement. The polarity correction factor or P_{pol} is deduced from [13]:

$$P_{pol} = \left| \frac{M_{raw}^+ - M_{raw}^-}{2M_{raw}} \right| \quad (2.11)$$

where, M_{raw}^+ and M_{raw}^- are the electrometer readings when the positive and negative charge is collected. M_{raw} can be either of the two and is used for reference dosimetry measurements. For large backgrounds, there can be slight difference in the two readings but usually the readings are similar with opposite polarity. The observations for calculating the polarity effects are recorded in table 2.4.

Table 2.5: The back scatter values corresponding to the HVL of 2.63 mm Al, interpolated from the data taken from the AAPM protocol [13]

Diameter (cm)	Back scatter (B_w)
1	1.0573
2	1.1145
3	1.1544
5	1.2092
10	1.2804
15	1.3101

The P_{pol} value was calculated to be 1.0028.

The calibration coefficient for air kerma, N_k was taken from the orthovoltage calibration report of the ionization chamber and the electrometer used in the calibration. The N_k value corresponding to the 100 kVp tube potential and 2.50 mm Al was $1.046 \frac{Gy}{Gy}$.

2.2.4 Backscatter factors

Data to calculate water kerma based backscatter factors was taken from the AAPM protocol, as a function of SSD, field size and HVL. Figure 2.7 is a plot of backscatter factors and HVL (mm AL) for different field sizes.

From the equations of exponential fit, the back scatter values corresponding to the HVL of 2.63 mm AL were calculated and plotted as a function of field size. Table 2.5 shows the calculated values of backscatter factors corresponding to the HVL of 2.63 mm AL.

Figure 2.8 is a plot of backscatter factors Vs the field size for 2.63 mm Al HVL. Corresponding to the field size of 8 cm, the back scatter factor was calculated from the equation of the exponential fit obtained from the plot. B_w was

Figure 2.7: Plot of water kerma based back scatter as a function of HVL (mm Al) for different field sizes [13]. Backscatter factors are the water-kerma ratio (i.e. unitless).

Table 2.6: The corrected electrometer readings for different radiation times

Time (sec)	Reading (actual)	Corrected reading	Absorbed dose(Gy)
22	0.4460	0.4421	0.5843
33	0.6998	0.6937	0.9168
44	0.9569	0.9486	1.2536
55	1.2110	1.2004	1.5865
60	1.3260	1.3144	1.7372

calculated to be 1.2635. For absolute accuracy, the BSF should be calculated using full Monte Carlo for the size of a mouse, taking into account the cross-sectional area also. This would give a low BSF compared to the standard values taken from the protocol. The BSF calculated here is for the field size of 8 cm and is consistent with the previous work [14]. The mass energy absorption coefficient $[(\frac{\mu_{en}}{\rho})_{air}^w]_{air}$ was taken as unity since the absorbed dose was calculated on the surface of tumor.

The ionization chamber and the electrometer were again set up to record readings for different radiation times. Table 2.6 includes the actual reading of the electrometer as well as the corrected reading. The correction factors like P_{TP} , P_{pol} , P_{ion} , etc were included to calculate the corrected value. The absorbed dose at the surface was calculated using equation 2.8.

The plot of time vs the absorbed dose (cGy) is shown in fig 2.9. The best fit equation was deduced from the graph shown in fig 2.9 and timer delay was estimated to be 2.766 sec. For irradiating mice, lead shields with cutouts of different diameter were used to avoid radiation to the whole body of mouse other than the tumor. The back scatter factors for these cut outs were considered to calculate the time required to irradiate a mouse to deliver a dose of 400 cGy. The

Figure 2.8: Plot of water kerma based back scatter factors (BSF) as a function of field size (cm) corresponding to the HVL of 2.63 mm Al. The error bars are too small to be clearly visible. Backscatter factors are the water-kerma ratio (i.e. unitless).

Figure 2.9: Plot of time (sec) vs the absorbed dose (cGy). The timer delay was deduced from the equation of best fit. It was found to be 2.766 sec. The error bars are too small to be clearly visible.

Table 2.7: Back scatter factors interpolated for 2.63 mm Al and 8 cm diameter

Diameter (cm)	2.0 mm Al	2.63 mm Al	3.0 mm Al	$\frac{BSF(cutout)}{BSF(cone)}$
1	1.058	1.057	1.063	0.836
2	1.108	1.115	1.120	0.882
3	1.140	1.154	1.164	0.913
5	1.182	1.209	1.221	0.956
8	1.216	1.264	1.267	1.000
10	1.238	1.280	1.298	1.013

Table 2.8: The irradiation time for different cutout shields

Diameter (mm)	BSF ratio	time (sec)
6.3	0.8272	163
7.0	0.8299	162
7.8	0.8331	161
9.4	0.8392	160
10.9	0.8449	159
12.5	0.8508	158

time for irradiation with different lead shields was thus calculated as [14]:

$$time(s) = \frac{0.3293(Dose(cGy)) + 2.766}{\frac{BSF(cutout)}{BSF(8cm)}} \quad (2.12)$$

To calculate the BSF (cut out), back scatter factors for 2.63 mm Al and field size of 8 cm were interpolated from the data given in AAPM protocol and are tabulated in table 2.7. The plot for this data is shown in figure 2.10.

The equation of best fit was found from the plotted values of BSF ratio, which was used to calculate the BSF for lead shield cut outs of various diameters. Table 2.8 gives the BSF ratio and time required to radiate for different cutout shields to deliver a dose of 400 cGy.

Figure 2.10: The plot of BSF ratio vs the diameter (cm). Error bars are too small to be visible.

Chapter 3

Results

To determine the efficacy, tumor volume was measured three times a week for a period of five weeks, starting from the first day of treatment. The normal tissue toxicity was measured from two different parameters: body weight and leukocyte count. The body weights were measured two times a week. To count the number of leukocytes, blood samples from the tail vein of mice were taken once a week for five weeks. In the first part of the work, the TPT dose was the same as in the previous study: 12 mg/kg body weight [14]. But later, experiments were done with lower TPT dose (6 mg/kg) and fractionated X-radiation (4 Gy, four times in two weeks, separately). The results of the statistical analysis are tabulated for all time points for TPT and XR groups and their corresponding graphs of median values are also plotted. For simplicity, the correspondence between the phases (active or rest) and the four treatment times are summarized in table 3.1 and refer to all the results :

Table 3.1: Summary of the relationship between the phases (active or rest) and four different treatment times followed in the experiment

Time	HALO	Phase
9 a.m.	3 HALO	Early rest phase
3 p.m.	9 HALO	Late rest phase
9 p.m.	15 HALO	Early active period
3 a.m.	21 HALO	Late active period

3.1 Chronomodulation of Topotecan(12 mg/kg)

3.1.1 Tumor Volume

At 9 a.m. (3 HALO), significant difference in the tumor volumes of the treated group and its matched control was found on ten days: from day 11 to day 32, which was the last day of observation. A time was considered significantly different when the p-value was found to be less than 0.05. Median tumor volume for the control group and the mice treated at 9 a.m. are tabulated in table 3.2. The significantly different points are marked with an asterisk (*). The graph of median tumor volume for 9 a.m. time point, showing statistically significant points with an open symbol, is shown in fig 3.1.

For the group treated at 3 p.m. (9 HALO), significant difference between the treated group and its matched control was found on 8 days: from day 9 to day 25. Table 3.3 shows median tumor volumes along with the number of mice and achieved statistical significance on all days. Figure 3.2 is the plot of these tabulated results. Mice being nocturnal, their day and night cycles are reversed from humans. The 9 a.m. and 3 p.m. times are the rest phase/night cycle of mice.

In the early active phase of mice, at 9 p.m. (15 HALO), significant difference between the treated and the control group was found on 13 days: from day 4 to

day 32. The median tumor volumes, number of mice and the calculated p-values for this group are tabulated in table 3.4. The graph of these values is shown in fig 3.3. In the late active phase of mice, at 3 a.m. (21 HALO), significant difference was found on 12 different days: from day 7 to day 32. The results of the statistical analysis for this group is given in table 3.5. The graph is shown in fig 3.4.

Table 3.2: The statistical analysis results of tumor volume for mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO). The significantly different tumor volumes of the treated and untreated group are marked by “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	18	9	41.9	45.1	0.9795
2	18	9	60.9	80.4	0.9386
4	18	9	68.0	84.5	0.9385
7	18	8	107.4	75.4	0.3892
9	18	8	131.9	30.5	0.0553
11	18	8	165.8	53.7	*0.0212
14	18	8	230.3	33.4	*0.0245
16	18	8	299.4	26.2	*0.0157
18	16	8	257.7	14.9	*0.0078
21	12	8	240.8	31.4	*0.0279
23	12	7	408.2	23.4	*0.0100
25	12	7	501.2	26.2	*0.0078
28	8	6	590.9	35.7	*0.0080
30	7	6	294.1	31.4	*0.0140
32	7	6	368.2	40.2	*0.0221

Table 3.3: The statistical analysis results of tumor volume for mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO). The significantly different tumor volumes of the treated and untreated group are marked by “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	18	8	27.4	28.6	0.7600
2	18	8	35.5	45.8	0.9778
4	18	8	82.5	45.3	0.1013
7	18	8	86.5	52.6	0.0553
9	18	8	122.6	46.7	*0.0008
11	18	8	148.3	48.1	*0.0050
14	18	8	218.5	39.3	*0.0008
16	16	8	263.8	46.5	*0.0011
18	15	8	271.9	30.1	*0.0009
21	13	8	401.1	38.5	*0.0008
23	10	8	343.5	40.3	*0.0003
25	10	8	459.2	46.5	*0.0014
28	4	8	478.2	77.9	0.0727
30	3	8	236.1	72.7	0.2788
32	3	8	432.7	71.1	0.1333

Table 3.4: The statistical analysis results of tumor volume for mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO). The significantly different tumor volumes of the treated and untreated group are marked “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	9	43.9	26.2	0.2581
2	9	9	50.4	44.3	0.7304
4	9	8	66.7	38.7	*0.0025
7	9	8	79.2	39.1	*0.0206
9	9	8	121.1	43.5	*0.0003
11	9	8	80.9	20.1	*0.0079
14	9	8	152.8	38.3	*0.0002
16	9	8	184.6	35.6	*<0.0001
18	9	8	235.5	41.4	*<0.0001
21	8	8	269.8	61.2	*0.0003
23	8	8	282.4	42.3	*0.0002
25	8	8	345.8	59.3	*0.0002
28	4	8	403.9	56.2	*0.0040
30	4	8	664.1	74.5	*0.0040
32	4	8	726.7	65.4	*0.0040

Table 3.5: The statistical analysis results of tumor volume for mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO). The significantly different tumor volumes of the treated and untreated group are marked by *.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	9	68.7	36.4	0.3865
2	9	9	56.5	49.1	0.4894
4	9	9	67.4	57.3	0.1135
7	9	9	82.9	36.4	*0.0040
9	9	9	105.5	29.3	*0.0040
11	9	9	102.1	22.1	*<0.0001
14	9	9	264.5	38.3	*<0.0001
16	8	9	244.0	28.3	*<0.0001
18	8	9	287.3	33.5	*<0.0001
21	5	9	299.4	54.9	*0.0010
23	5	9	354.8	45.7	*0.0100
25	5	9	486.4	64.1	*0.0010
28	5	9	393.9	37.9	*0.0010
30	5	9	552.6	70.3	*0.0010
32	5	9	608.9	53.7	*0.0010

Figure 3.1: Median tumor volume of mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO) compared with the matched control

Figure 3.2: Median tumor volume of mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO) compared with the matched control

Figure 3.3: Median tumor volume of mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO) compared with the matched control

3AM (21 HALO)- Median tumor volume

Figure 3.4: Median tumor volume of mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO) compared with the matched control

3.1.2 Body Weight

The body weight measurements, number of mice left and the p-values, calculated using the Mann-Whitney test, for 9 a.m (3 HALO) time point are given in table 3.6. The corresponding graph is plotted in fig 3.5. From the p-values, no significant difference between the treated and the control group was found during the period of observation for this time.

The body weights for 3 p.m. (9 HALO) group are plotted in fig 3.6. The results of statistical analysis are tabulated in table 3.7. Significant difference between the treated and the control group was found on two different days: day 7 and day 14.

However, no significant difference was found on any of the days for the active period of mice (9 p.m. and 3 a.m.).

Table 3.6: The statistical analysis results of body weight for mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	18	9	24.2	24.3	0.7575
4	18	9	24.7	24.5	0.8370
7	18	8	25.0	24.8	0.8458
11	18	8	25.1	24.9	0.5784
14	18	8	25.2	24.9	0.1409
18	16	8	25.2	24.2	0.8783
21	12	8	25.2	24.9	0.9692
25	12	7	25.0	25.2	0.9663
28	8	6	25.0	25.9	0.2284
32	7	6	24.9	25.9	0.7308

Table 3.7: The statistical analysis results of body weight for mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	18	8	25.4	24.4	0.2911
4	18	8	25.3	24.1	0.0956
7	18	8	25.4	23.2	*0.0373
11	18	8	25.5	23.4	0.0710
14	18	8	25.8	23.7	*0.0245
18	15	8	25.7	23.7	0.0569
21	13	8	25.9	24.9	0.1924
25	10	8	25.8	23.7	0.2031
28	4	8	26.0	24.6	0.9333
32	3	8	25.7	23.8	0.6303

Table 3.8: The statistical analysis results of body weight for mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	9	24.4	24.3	0.9314
4	9	9	25.0	24.9	0.9626
7	9	9	24.8	24.0	0.4807
11	9	9	25.5	25.5	0.8148
14	9	9	25.9	25.0	0.3213
18	9	9	25.6	25.0	0.7430
21	8	9	26.3	25.3	0.7209
25	8	9	26.2	25.0	0.9591
28	4	9	27.1	26.0	0.5697
32	4	8	26.9	26.0	0.4962

Table 3.9: The statistical analysis results of body weight for mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	9	24.0	24.3	0.3865
4	9	9	25.3	24.8	0.9314
7	9	9	24.4	24.2	0.7962
11	9	9	24.5	24.6	0.9314
14	9	9	25.4	25.3	0.9314
18	8	9	24.7	25.2	0.3704
21	5	9	24.7	26.1	0.1898
25	5	9	24.3	25.6	0.1898
28	5	9	25.3	25.4	0.6064
32	5	9	25.7	25.8	0.6993

Figure 3.5: Median body weight of mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO) compared with their matched control

Figure 3.6: Median body weight of mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO) compared with their matched control

Figure 3.7: Median body weight of mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO) compared with their matched control

Figure 3.8: Median body weight of mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO) compared with their matched control

3.1.3 Leukocyte count

In the rest phase of mice, statistically significant difference between the treated and the control group was observed on some days. At 9 a.m. (3 HALO), significant difference in the leukocyte counts was observed on two different days: day 3 and day 10. The calculated p-values, along with the leukocyte count and mice number, is tabulated in table 3.10. The graph for these values is shown in fig 3.9. At 3 p.m. (9 HALO), significant difference was observed on day 10 only. The probability values for this group are given in table 3.11. The graph is plotted in fig 3.10.

In the active phase of mice, at 9 p.m. and 3 a.m., no significant difference between the treated and untreated group was found. The p-values are shown in tables 3.12 and 3.13 respectively. The graphs for the respective time points are shown in fig 3.11 and 3.12.

Table 3.10: Results of the statistical analysis results of leukocyte count for mice treated TPT (12 mg/kg) at 9 a.m. (3 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/ mm^3)		p-value ($p < 0.05$)
	Control	TPT	Control	TPT	
3	18	9	9550	5950	*0.0126
10	18	8	8750	5825	*0.0169
17	17	8	8100	8925	0.7487
24	12	7	11000	9700	0.3104
31	7	6	8700	9200	0.8357

Table 3.11: Results of the statistical analysis of leukocyte count for mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	TPT	Control	TPT	
3	18	8	9300	11825	0.1198
10	18	8	8450	4475	*0.0025
17	16	8	7450	9350	0.3123
24	10	8	10575	9875	0.7618
31	3	8	12750	9750	0.9212

Table 3.12: Results of the statistical analysis of leukocyte count for mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	TPT	Control	TPT	
3	9	8	4400	3500	0.2766
10	9	8	6000	5575	0.7430
17	9	8	6050	6075	0.4234
24	8	8	5650	7075	0.3823
31	4	8	6050	6750	0.2828

Table 3.13: Results of the statistical analysis of leukocyte count for mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	TPT	Control	TPT	
3	9	9	3200	3800	0.8633
10	9	9	3500	2800	0.5457
17	8	9	4375	5550	0.4234
24	5	9	4950	4550	0.4376
31	5	9	5050	5800	0.1898

Figure 3.9: Leukocyte count of mice treated with TPT (12 mg/kg) at 9 a.m. (3 HALO) compared with their matched control

Figure 3.10: Leukocyte count of mice treated with TPT (12 mg/kg) at 3 p.m. (9 HALO) compared with their matched control

Figure 3.11: Leukocyte count of mice treated with TPT (12 mg/kg) at 9 p.m. (15 HALO) compared with their matched control

Figure 3.12: Leukocyte count of mice treated with TPT (12 mg/kg) at 3 a.m. (21 HALO) compared with their matched control

3.2 Chronomodulation of Topotecan(6 mg/kg) and X-Radiation (4 Gy/fraction)

3.2.1 Tumor volume

At 9 a.m. (3 HALO), the group treated with TPT (6 mg/kg) was significantly different from its matched control on day 0 of the treatment. In the group treated with XR (4 Gy/fraction), significant difference between the treated and the control group was observed on day 23 of the treatment. The p-values calculated for both TPT and XR treated groups are tabulated in tables 3.14 and 3.18. The plot of the analyzed values is shown in fig 3.13.

At 3 p.m. (9 HALO), the TPT treated group showed significant difference in tumor volumes from its control group on 7 days: day 11 to day 25. In the XR treated group, significant difference was observed on 8 different days: day 4, day 11 to day 25. The median tumor volumes, number of mice and the p-values for TPT as well as the XR group are given in tables 3.15 and 3.19. The graph of median tumor volumes versus the number of days since first treatment is shown in fig 3.14. The significantly different points for both groups are plotted as open symbols.

In the early active phase of mice, at 9 p.m.(15 HALO), the mice treated with TPT showed significant difference on 4 days: day 16, 21, 28 and 32. The XR treated group showed significant difference on 9 days: day 9, day 16 to day 32. The p-values for both these groups are given in tables 3.16 and 3.20. The graph for the tabulated values is shown in fig 3.15.

In the late active period of mice, at 3 a.m. (21 HALO), there was significant

difference in the TPT group on 10 different days: day 9, day 14 to day 32. In the XR group, significant difference was observed on 8 days: day 0, day 4 to day 9, day 23, 25, 30 and 32. The median tumor volumes and their statistical significance is shown in tables 3.14 and 3.18. The corresponding graph is plotted in fig 3.16.

Table 3.14: The statistical analysis results of tumor volume for mice treated with TPT (6mg/kg) at 9 a.m. (3 HALO). The significantly different tumor volumes of the treated and untreated group are marked by “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	18	16	41.9	70.5	*0.0218
2	18	16	60.9	68.7	0.3606
4	18	16	68.0	129.5	0.0815
7	18	16	107.4	143.5	0.8495
9	18	16	131.9	89.6	0.5233
11	18	16	165.8	74.1	0.3427
14	18	16	230.3	144.1	0.3980
16	18	15	299.4	95.9	0.0627
18	16	13	257.7	196.2	0.6770
21	12	13	340.8	127.4	0.2211
23	12	13	408.2	160.3	0.4628
25	12	13	501.4	259.6	0.7237
28	8	13	590.9	147.2	0.7445
30	7	13	294.1	200.0	0.8121
32	7	13	368.2	314.5	0.6345

Table 3.15: The statistical analysis results of tumor volume for mice treated with TPT (6mg/kg) at 3 p.m. (9 HALO). The significantly different tumor volumes of the treated and untreated group are marked by “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	18	8	27.4	36.1	0.5011
2	18	8	35.5	45.6	0.7431
4	18	8	82.5	93.5	0.9587
7	18	8	86.5	73.3	0.5928
9	18	8	122.6	63.9	0.0578
11	18	8	148.3	66.8	*0.0181
14	18	8	218.5	84.3	*0.0036
16	16	8	263.8	84.5	*0.0015
18	15	8	271.9	95.1	*0.0129
21	13	8	401.1	92.7	*0.0017
23	10	8	343.5	124.8	*0.0051
25	10	8	459.2	127.9	*0.0051
28	4	8	478.2	134.4	0.0644
30	3	8	236.1	80.9	0.2864
32	3	8	432.7	185.4	0.2843

Table 3.16: The statistical analysis results of tumor volume for mice treated with TPT (6 mg/kg) at 9 p.m. (15 HALO). The significantly different tumor volumes of the treated and untreated group are marked by “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	16	43.9	35.9	0.9774
2	9	16	50.4	56.3	0.5907
4	9	16	66.7	76.2	0.6711
7	9	16	79.2	95.3	0.8875
9	9	16	121.1	79.9	0.2236
11	9	16	80.9	142.6	0.1067
14	9	16	152.8	103.5	0.0579
16	9	16	184.6	88.4	*0.0445
18	9	15	235.5	178.1	0.2331
21	8	15	269.8	137.3	*0.0185
23	8	15	282.4	159.1	0.0759
25	8	14	345.8	203.6	0.0606
28	4	14	403.9	195.9	*0.0169
30	4	12	664.1	228.1	0.0602
32	4	12	726.7	334.7	*0.0454

Table 3.17: The statistical analysis results of tumor volume for mice treated with TPT (6 mg/kg) at 3 a.m. (21 HALO). The significantly different tumor volumes of the treated and untreated group are marked by *.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	16	68.7	54.7	0.9323
2	9	16	56.5	57.5	0.5522
4	9	16	67.4	75.6	0.6304
7	9	16	82.9	86.7	0.2236
9	9	16	105.5	73.8	*0.0162
11	9	16	102.1	107.7	0.4117
14	9	15	264.5	60.4	*0.0010
16	8	15	244.0	70.7	*0.0004
18	8	15	287.3	116.7	*0.0007
21	5	15	299.4	92.9	*0.0012
23	5	14	354.8	118.0	*0.0014
25	5	14	486.4	144.8	*0.0014
28	5	14	393.9	111.4	*0.0047
30	5	14	552.6	147.5	*0.0014
32	5	14	608.9	128.5	*0.0014

Table 3.18: The statistical analysis results of tumor volume for mice treated with XR (4Gy/fraction) at 9 a.m. (3 HALO). The significantly different tumor volumes of the treated and untreated group are marked by “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	XR	Control	XR	
0	18	14	41.9	64.5	0.1336
2	18	14	60.9	80.9	0.3721
4	18	14	68.0	86.3	0.8345
7	18	14	107.4	106.1	0.9848
9	18	14	131.9	131.9	0.7182
11	18	14	165.8	106.1	0.1437
14	18	14	230.3	123.6	0.4141
16	18	14	299.4	144.3	0.1656
18	16	14	257.7	142.9	0.1638
21	12	14	340.8	166.9	0.2472
23	12	14	408.2	142.4	*0.0422
25	12	13	501.4	232.8	0.1827
28	8	13	590.9	216.3	0.1804
30	7	12	294.1	153.8	0.8991
32	7	12	368.2	60.7	0.4221

Table 3.19: The statistical analysis results of tumor volume for mice treated with XR (4Gy/fraction) at 3 p.m. (9 HALO). The significantly different tumor volumes of the treated and untreated group is marked by “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	XR	Control	XR	
0	18	14	27.4	40.7	0.9848
2	18	14	35.5	33.8	0.7468
4	18	14	82.5	44.9	*0.0385
7	18	13	86.5	63.9	0.0817
9	18	13	122.6	96.2	0.0573
11	18	13	148.3	60.7	*0.0139
14	18	13	218.5	78.6	*0.0029
16	16	13	263.8	88.4	*0.0027
18	15	13	271.9	90.3	*0.0188
21	13	13	401.1	131.3	*0.0104
23	10	13	343.5	135.1	*0.0058
25	10	13	459.2	170.6	*0.0170
28	4	13	478.2	275.2	0.1929
30	3	12	236.1	201.6	0.8286
32	3	12	432.7	225.1	0.5160

Table 3.20: The statistical analysis results of tumor volume for mice treated with XR (4Gy/fraction) at 9 p.m. (15 HALO). The significantly different tumor volumes of the treated and untreated group is marked by “*”.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	XR	Control	XR	
0	9	14	43.9	19.3	0.3611
2	9	14	50.4	19.1	0.3289
4	9	14	66.7	43.5	0.3289
7	9	14	79.2	32.2	0.0632
9	9	13	121.1	37.3	*0.0062
11	9	13	80.9	67.5	0.8412
14	9	13	152.8	86.4	0.0951
16	9	13	184.6	66.1	*0.0452
18	9	13	235.5	90.0	*0.0232
21	8	13	269.8	136.6	*0.0391
23	8	12	282.4	98.5	*0.0062
25	8	12	345.8	111.2	*0.0038
28	4	12	403.9	155.9	*0.0455
30	4	12	664.1	117.3	*0.0130
32	4	12	726.7	166.3	*0.0044

Table 3.21: The statistical analysis results of tumor volume for mice treated with XR (4Gy/fraction) at 3 a.m. (21 HALO). The significantly different tumor volumes of the treated and untreated group are marked by *.

Day	Number of mice		Median tumor volume (mm^3)		p-value (p<0.05)
	Control	XR	Control	XR	
0	9	14	68.7	22.9	*0.0153
2	9	14	56.5	33.4	0.0632
4	9	14	67.4	40.4	*0.0407
7	9	14	82.9	53.1	*0.0407
9	9	14	105.5	39.6	*0.0407
11	9	14	102.1	96.4	0.5496
14	9	14	264.5	110.4	0.0951
16	8	14	244.0	126.7	0.0818
18	8	14	287.3	137.2	0.0518
21	5	14	299.4	150.1	0.0868
23	5	14	354.8	157.9	*0.0109
25	5	14	486.4	245.1	*0.0142
28	5	14	393.9	307.6	0.3792
30	5	14	552.6	319.9	*0.0063
32	5	14	608.9	343.9	*0.0014

Figure 3.13: Tumor volume of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 a.m. (3 HALO) compared with their matched control

Figure 3.14: Tumor volume of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 p.m. (9 HALO) compared with their matched control

Figure 3.15: Tumor volume of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 p.m. (15 HALO) compared with their matched control

Figure 3.16: Tumor volume of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 a.m. (21 HALO) compared with their matched control

3.2.2 Body Weight

Body weight were measured to observe the normal tissue toxicity. Decrease in the body weight during the period of observation would suggest toxic effects of the treatment agent. At 9 a.m., in the TPT group, significant difference was observed on day 0, which was the first day of treatment. The body weights of the XR treated group were significantly different from its control group on all the days of observation i.e. from day 0 to day 32. The p-values for the TPT and XR group are given in tables 3.22 and 3.26. The plot of median body weights is shown in fig 3.17.

At 3 p.m., the TPT group showed no difference from its control group on any of the days, while the XR group differed on all days of the experiment. The median body weights, number of mice left since first day of treatment and the statistical values are shown in tables 3.23 and 3.27.

At 9 p.m., the body weights of the TPT group showed statistical significance on 3 days: day 0,7 and 25. The XR group did not show any difference on any of the days. The median body weights for this group are shown in tables 3.24 and 3.36. The graph for the calculated values is shown in fig 3.18.

At 3 a.m., significant difference in the body weights of the treated and the control group was observed on all days i.e. from day 0 to day 32. The XR group showed difference on day 0 of the treatment. The p-values for TPT and XR group are given in tables 3.25 and 3.29. The corresponding graph is plotted in fig 3.20.

Table 3.22: The statistical analysis results of body weight for mice treated with TPT (6 mg/kg) at 9 a.m. (3 HALO). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	18	16	24.2	26.1	*0.0012
4	18	16	24.7	25.8	0.0814
7	18	16	25.0	24.9	0.5927
11	18	16	25.1	25.1	0.4582
14	18	16	25.2	25.2	0.7561
18	16	13	25.2	25.3	0.1883
21	12	13	25.2	26.2	0.0917
25	12	13	25.0	25.9	0.0971
28	8	13	25.0	26.4	0.0648
32	7	13	24.9	25.4	0.5005

Table 3.23: The statistical analysis results of body weight for mice treated with TPT (6 mg/kg) at 3 p.m. (9 HALO). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	18	16	25.4	24.4	0.2695
4	18	16	25.3	24.1	0.5460
7	18	16	25.4	23.2	0.3340
11	18	16	25.5	23.4	0.6662
14	18	16	25.8	23.7	0.1206
18	15	15	25.7	23.7	0.3194
21	13	15	25.9	24.9	0.9449
25	10	15	25.8	23.7	0.8897
28	4	15	26.0	24.6	0.5484
32	3	14	25.7	23.8	0.5705

Table 3.24: The statistical analysis results of body weight for mice treated at 9 p.m. (15 HALO) with TPT (6 mg/kg). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	16	24.4	27.7	*0.0109
4	9	16	25.0	26.4	0.0792
7	9	16	24.8	26.8	*0.0388
11	9	16	25.5	26.6	0.1834
14	9	16	25.9	26.6	0.2697
18	9	15	25.6	26.8	0.1525
21	8	15	26.3	27.0	0.3328
25	8	14	26.2	28.2	*0.0478
28	4	14	27.1	28.3	0.5239
32	4	13	26.9	27.4	0.9517

Table 3.25: The statistical analysis results of body weight for mice treated at 3 a.m. (21 HALO) with TPT (6 mg/kg). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	16	24.0	28.4	*<0.001
4	9	16	25.3	27.6	*0.0011
7	9	16	24.4	27.3	*0.0030
11	9	16	24.5	27.1	*0.0085
14	9	15	25.4	27.1	*0.0171
18	8	15	24.7	28.2	*0.0006
21	5	15	24.7	28.8	*0.0030
25	5	14	24.3	29.2	*0.0031
28	5	14	25.3	29.2	*0.0063
32	5	14	25.7	28.7	*0.0160

Table 3.26: The statistical analysis results of body weight for mice treated at 9 a.m. (3 HALO) with XR (4 Gy/fraction). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	XR	Control	XR	
0	18	14	24.2	26.7	*0.0001
4	18	14	24.7	26.6	*0.0001
7	18	14	25.0	27.6	*<0.0001
11	18	14	25.1	26.6	*0.0059
14	18	14	25.2	28.4	*0.0001
18	16	14	25.2	27.6	*0.0002
21	12	14	25.2	27.9	*0.0003
25	12	13	25.0	27.7	*0.0008
28	8	13	25.0	28.3	*0.0004
32	7	12	24.9	27.9	*0.0002

Table 3.27: The statistical analysis results of body weight for mice treated at 3 p.m. (9 HALO) with XR (4 Gy/fraction). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	XR	Control	XR	
0	18	14	25.4	28.0	*0.0005
4	18	14	25.3	28.3	*0.0007
7	18	13	25.4	29.1	*<0.0001
11	18	13	25.5	28.3	*0.0003
14	18	13	25.8	29.4	*<0.0001
18	15	13	25.7	27.8	*0.0011
21	13	13	25.9	29.0	*0.0004
25	10	13	25.8	28.8	*0.0010
28	4	13	26.0	29.3	*0.0150
32	3	12	25.7	28.2	*0.0209

Table 3.28: The statistical analysis results of body weight for mice treated at 9 p.m. (15 HALO) with XR (4 Gy/fraction). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	XR	Control	XR	
0	9	14	24.4	25.1	0.8997
4	9	14	25.0	25.1	0.5495
7	9	14	24.8	24.3	0.6365
11	9	13	25.5	24.9	0.1511
14	9	13	25.9	25.3	0.5257
18	9	13	25.6	25.8	0.8937
21	8	13	26.3	26.2	0.2320
25	8	12	26.2	25.9	0.8773
28	4	12	27.1	26.0	0.2030
32	4	12	26.9	26.5	0.3309

Table 3.29: The statistical analysis results of body weight for mice treated at 3 a.m. (21 HALO) with XR (4 Gy/fraction). The significantly different body weights of the treated and untreated group are starred.

Day	Number of mice		Median body weights (g)		p-value (p<0.05)
	Control	TPT	Control	TPT	
0	9	14	24.0	25.6	*0.0438
4	9	14	25.3	25.0	0.9498
7	9	14	24.4	24.6	0.6591
11	9	14	24.5	25.2	0.7054
14	9	14	25.4	25.0	0.7289
18	8	14	24.7	24.7	0.6572
21	5	14	24.7	25.2	0.7459
25	5	14	24.3	25.6	0.2663
28	5	14	25.3	26.1	0.4588
32	5	14	25.7	25.8	0.8530

Figure 3.17: Median body weight of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 a.m. (3 HALO) compared with their matched control

Figure 3.18: Median body weight of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 p.m. (9 HALO) compared with their matched control

Figure 3.19: Median body weight of mice treated with TPT (6 mg/kg) and XR (6 Gy/fraction) at 9 p.m. (15 HALO) compared with their matched control

Figure 3.20: Median body weight of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 a.m. (21 HALO) compared with their matched control

3.2.3 Leukocyte count

Second parameter to observe normal tissue toxicity was the reduction in peripheral blood leukocyte count. At 9 a.m., the TPT group showed significant difference on 3 different days: from day 3 to 17. The XR group also showed significant difference on 3 days: day 17 to 31. The calculated probability for both groups is given in tables 3.30 and 3.34. The graph showing significantly different points is shown in fig 3.21.

At 3 p.m., the TPT group showed toxic effects on all the days when blood sample was taken i.e. day 3, 10, 17, 24 and 31. The XR group showed difference on day 10, 17 and 31. The p-values are given in tables 3.31 and 3.35. The graph is shown in fig 3.22.

The active period of mice did not show much toxic effects. At 9 p.m., the TPT group showed significant difference on day 31 of the treatment. In the XR group, significant difference was observed on day 17. The statistical values for these two groups are given in tables 3.32 and 3.35. The graph is plotted in fig 3.23.

At 3 a.m., the TPT group did not show any significant difference on any day of the observation, while the XR group showed difference on day 10 of the treatment. The median leukocyte count, number of mice left since first day of treatment and the statistical values for these groups are given in table 3.33 and 3.37. The graph is plotted in fig 3.24.

Table 3.30: The statistical analysis results of leukocyte count for mice treated with TPT (6 mg/kg) at 9 a.m. (3 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	TPT	Control	TPT	
3	18	15	9550	5550	*0.0005
10	18	15	8750	6100	*0.0079
17	17	13	8100	5750	*0.0469
24	12	13	11000	8800	0.1573
31	7	13	8700	6450	0.0962

Table 3.31: The statistical analysis results of leukocyte count for mice treated with TPT (6 mg/kg) at 3 p.m. (9 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	TPT	Control	TPT	
3	18	16	9300	5025	*0.0042
10	18	16	8450	5200	*<0.0001
17	16	15	7450	4650	*0.0035
24	10	15	10575	5650	*0.0036
31	3	15	12750	6000	*0.0129

Table 3.32: The statistical analysis results of leukocyte count for mice treated with TPT (6 mg/kg) at 9 p.m. (15 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	TPT	Control	TPT	
3	9	16	4400	4100	0.9774
10	9	16	6000	5050	0.8874
17	9	15	6050	4400	0.0838
24	8	15	5650	4850	0.6283
31	4	14	6050	3875	*0.0076

Table 3.33: The statistical analysis results of leukocyte count for mice treated with TPT (6 mg/kg) at 3 a.m. (21 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	TPT	Control	TPT	
3	9	16	3200	4375	0.2458
10	9	16	3500	4025	0.3802
17	8	15	4375	4450	0.6283
24	5	14	4950	4700	0.6434
31	5	14	5050	4225	0.6770

Table 3.34: The statistical analysis results of leukocyte count for mice treated with XR (4 Gy/fraction) at 9 a.m. (3 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	XR	Control	XR	
3	18	14	9550	6825	0.0712
10	18	14	8750	7000	0.1489
17	17	14	8100	4550	*<0.0001
24	12	13	11000	4500	*0.0010
31	7	13	8700	6400	*0.0433

Table 3.35: The statistical analysis results of leukocyte count for mice treated with XR (4 Gy/fraction) at 3 p.m. (9 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	XR	Control	XR	
3	18	14	9300	6350	0.0627
10	18	13	8450	6100	*0.0073
17	16	13	7450	4450	*0.0002
24	10	13	10575	5850	0.0882
31	3	12	12750	5050	*0.0364

Table 3.36: The statistical analysis results of leukocyte count for mice treated with XR (4 Gy/fraction) at 9 p.m. (15 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	XR	Control	XR	
3	9	14	4400	4425	0.6143
10	9	13	6000	4200	0.2854
17	9	12	6050	3450	*0.0276
24	8	12	5650	3625	0.4636
31	4	11	6050	4000	0.2149

Table 3.37: The statistical analysis results of leukocyte count for mice treated with XR (4 Gy/fraction) at 3 a.m. (21 HALO). The significantly different leukocyte counts of the treated and the control group are starred.

Day	Number of mice		Median Leukocyte counts (leukocytes/mm ³)		p-value (p<0.05)
	Control	XR	Control	XR	
3	9	14	3200	4300	0.1306
10	9	14	3500	4150	*0.0275
17	8	14	4375	3350	0.1247
24	5	14	4950	3200	0.5473
31	5	13	5050	3300	0.6221

Figure 3.21: Leukocyte count of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 a.m. (3 HALO) compared with their matched control

Figure 3.22: Leukocyte count of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 p.m. (9 HALO) compared with their matched control

Figure 3.23: Leukocyte count of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 9 p.m. (15 HALO) compared with their matched control

Figure 3.24: Leukocyte count of mice treated with TPT (6 mg/kg) and XR (4 Gy/fraction) at 3 a.m. (21 HALO) compared with their matched control

Chapter 4

Analysis

4.1 Chronomodulation of TPT (12 mg/kg)

The statistical analysis using Mann-Whitney test revealed significant differences between the treated group and their respective controls. In the 9 a.m. group, the tumor volumes of the treated mice differed from the control mice on 10 days: day 11, 14, 16, 18, 21, 23, 25, 28, 30, and 32. The 3 p.m. group differed on 8 days: day 9, 11, 14, 16, 18, 21, 23, and 25. The light shifted mice in the 9 p.m. group showed significant difference on 13 days: day 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 30, and 32. The 3 a.m. group differed on 12 days: day 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 30, and 32. From these observations, it is clear that the active phase mice were able to regress the tumor within the first week of the treatment. The rest phase mice achieved this regression after three treatments i.e. in the second week. There are few points worth mentioning here: The mice in the late active period (3 a.m.) and early rest period (9 a.m.) had comparatively higher tumor volumes. But, for all four time points, the tumor volumes of the TPT and the control

group were comparable. In the active phase mice, the 3 a.m. group started off with higher tumor volumes than the 9 p.m. group, but significant difference was achieved by day 7 of the treatment. From the number of days, when the treated group differed significantly from its matched control, it is clear that the tumor efficacy was better achieved in the active phase of mice. The difference among all four time points was, however, not sufficient to conclude that the treatment at any particular time was more effective compared to the others.

The median body weights of mice at all time points in the treated and the control group were comparable. The Mann-whitney test revealed significant difference between the treated and the control group for only 3 p.m. (late rest phase) time point. Since this parameter was used to observe toxic effects to the normal tissue, the treatment did not show any signs of toxicity in the 9 a.m., 9 p.m. and 3 a.m. groups.

The second parameter to observe toxic effects to the normal tissue was the number of leukocytes present in the peripheral blood. The rest phase mice showed toxicity on 2 days (day 3 and 10) for the 9 a.m. group and on 1 day (day 10) in the 3 p.m. group. Since this was observed during the time when the treatment was actually delivered, in the first two weeks of the experiment, it could be attributed to the toxic effects of TPT. Once the treatment was over, mice seemed to regain their leukocyte count and no significant difference was observed thereafter. The active phase mice did not show any toxic effects, as observed from their leukocyte counts.

Even though the TPT dose of 12 mg/kg was able to effectively reduce tumor volumes in all four time points, the normal tissue toxicity was observed only in the rest phase mice. From the body weight and the leukocyte count, mice treated

during the active phase (9 p.m. and 3 a.m.) were able to tolerate the treatment agent better than the rest phase mice.

Thus, from the tumor volumes, all four times had significant effect in reducing the tumor volume. But, it is not clear if there were any significant differences among the four time points. The body weight and leukocyte count showed signs of toxicity in the 3 p.m. group. This is in general agreement with the p53 +/+ results in that the active phase mice (9 a.m. and 3 p.m.) are associated with least normal tissue toxicity, while the rest phase mice (in this study, late rest phase particularly) were associated with most normal tissue toxicity as per the significant differences in the body weight and leukocyte count.

4.2 Chronomodulation of TPT (6 mg/kg) and XR (4 Gy/fraction)

4.2.1 Tumor volume

The tumor volumes of the mice treated with TPT at 9 a.m. showed significant difference on day 0, which was the first day of treatment. This difference was due to the fact that tumor volume of the TPT group were higher than its control group at the beginning of treatment. It was not related to the effectiveness of the treatment agent. At 3 p.m., significant difference was seen on 7 days: day 11, 14, 16, 18, 21, 23, and 25. At 9 p.m., significant difference was seen on 4 different days: day 16, 21, 28, and 32. At 3 a.m., significant difference was observed on 10 days: day 9, 14, 16, 18, 21, 23, 25, 28, 30, and 32. The effect of TPT in controlling tumor growth was seen after three treatments delivered on days 0, 4,

and 7 for 3 p.m. and 3 a.m. groups. From table 3.14 to 3.17, it was observed that the tumor volumes of the TPT and the respective controls in the late active period (3 a.m.) and early rest period (9 p.m.) were heavier than the other two groups. The tumor volumes of control and TPT groups were comparable at the beginning of treatment for all time points. From the number of days when we achieved a significant p-value, the 3 p.m. and 3 a.m. time points for the TPT group seem to be quite effective in regressing the tumor.

The results for the TPT group are somewhat similar to the previous study, except that the tumor regression in this study was also seen for mice treated at 3 pm. Circadian dependent changes like blood flow, and liver metabolism play an important part in determining the best tolerated dose. Studies on the pharmacokinetics of 5-FU have shown that dehydropyrimidine dehydrogenase (DPD), an enzyme that limits the rate of catabolic activity of 5-FU, peaked near 3 HALO (early rest period of mice)[2]. In yet another study on the sarcoma-bearing rats, the circadian rhythms in the tumor blood flow were found to vary with the proliferation activity. The tumor blood flow was maximum at the time of high proliferation. The anti-tumor activity of doxorubicin, injected intravenous, peaked at 16 HALO when the tumor blood flow was maximum. This dosing time also had minimum toxic effects. The increase in tumor blood flow could have contributed in effectively regressing the tumor when TPT was administered at 3 a.m. by increasing the drug delivery during the active period of mice [7].

The mice treated with X-radiation also showed results similar to the TPT group. The median tumor volumes of the mice in late active period (3 a.m.) and early rest period were higher than the mice in late rest period (3 p.m.) and early active period (9 p.m.). At 9 a.m., significant difference was observed on day 23 of the

treatment. In the late rest period (3 p.m.), tumor regression was seen on 8 days: day 4, 11, 14, 16, 18, 21, 23, and 25. At 9 p.m., significant difference in the median tumor volumes of the treated and the control group was observed on 8 days: day 9, 18, 21, 23, 25, 28, 30, and 32. At 3 a.m., significant differences were observed on 9 days: day 0, 4, 7, 9, 18, 23, 25, 30, and 32. Since day 0 is the first day of treatment, the significant value that was observed for 3 a.m. group was not due to the XR treatment. The tumor volume of the XR group were comparatively smaller than their respective control group. From the number of days of marked significance, the 3 p.m., 9 p.m. and 3 a.m. time points of the XR group seem to be quite effective in regressing tumor. The active period of mice clearly had an edge over the treatment delivered during their rest phase. The increased therapeutic effect could be due to the increase in proliferation of cells during the active period and actively proliferating cells are known to be very sensitive to the radiation treatment [7].

4.2.2 Body weight

The body weights of the mice treated with TPT did not show any significant difference for the mice in the rest phase. The mice in the TPT and control groups of 9 a.m. and 3 p.m. time points had comparable body weight at the beginning of the treatment. Significant difference was observed on day 0 of the 9 a.m. group. Since it was the first day of treatment, this difference was not due to any side effects of TPT. It could be a random occurrence and not attributed to the normal tissue toxicity. The mice treated at 9 p.m. showed significant difference on 3 days: day 0, 7, and 25. The difference on day 0 was, again, not due to the toxic effects of the drug. The mice in the TPT group of 9 p.m. weighed more

than the control mice. The difference on day 25 is another random occurrence and probably not due to the toxic effects of TPT. There was an increase in the weight of the TPT group on day 25. The treated group maintained a stable weight through out the experiment. Any variation that occurred was within 3 % of the weight on day 0, indicating minimal toxic effects, if there were any. At 3 a.m., significant differences were seen on all days: day 0 to day 32. In this group, there was a stark difference (almost 20 %) between the weights of the treated and the control group at the beginning of the treatment. This difference was maintained throughout the experiment. There was approximately 5 % drop in the weight of mice during the period of drug administration i.e. day 0, 4, 7, and 11. This could be due to the side effects of TPT. Once the treatment was over, the mice regained their initial weight and increasing trend was seen afterwards. The increase in the weight of treated group during the latter half of the experiment also lead to the significant differences in both groups (treated and control).

In the XR group treated at 9 a.m. and 3 p.m., significant difference between the control and treated group was observed on all the days: day 0 to day 32. The XR group weighed approximately 10 % more than the control group at both these time points. The significant difference that we see here is because of the higher weights of XR group. Any reduction in weight could have been attributed to the toxic effects of irradiation. The drop in weight was seen around days 11, 18, 32. For 9 a.m. time point, the drop in weight on days 11 and 18 could have been the after effects of the irradiation treatment delivered on days 0, 4, 7, and 11. Once the treatment was over, the mice maintained a stable weight. Thus, minimal toxic effects due to irradiation were observed at this time point. Similar pattern of drop in the body weight was observed for 3 p.m. group. There was

decrease in the body weight on day 11, 18 and 32. The body weights of the mice treated at 9 p.m. and 3 a.m. had weights comparable to their matched controls. No significant difference was observed for the 9 p.m. group. The difference on day 0 of the 3 a.m. group is not due to the toxic effects because this is the first day of treatment.

4.2.3 Leukocyte count

The leukocyte counts of the mice treated with TPT at 9 a.m. showed significant difference on 3 days: day 3, 10 and 17. These days coincide with the time when TPT was administered. Once the treatment was over, the toxic effects also subsided. At 3 p.m., significant difference was observed on all the days when blood sample was taken, indicating maximum toxicity at this time. At 9 p.m., there was only one day, day 31, when any difference was observed. Mice had otherwise maintained a stable number of leukocytes through out the experiment. At 3 a.m., no significant difference was observed, indicating least toxicity.

The XR group at 9 a.m. showed toxicity on 3 days: day 17, 24, and 31. The toxic effects of the irradiation were seen after the treatment was delivered. At 3 p.m., the significant variation was seen on days 10, 17 and 31. In the early active period, there was difference on day 17 and in the late active period, it was day 10. Both these times coincide with the time of treatment administration and could have been due to the toxic effects of irradiation.

The TPT group had showed marked tumor efficacy at 3 p.m. and 3 a.m. time points. The normal tissue toxicity, as observed from the body weight was maximum for the 3 a.m. group and minimum for 3 p.m. The leukocyte results indicated the opposite: maximum toxicity at 3 p.m. and minimum toxicity at 3

a.m. Therefore, it was not possible to conclude from this work whether the p53 -/- tumors behaved similar to the p53 +/+ tumors with TPT chronotherapy.

In the XR group, maximum tumor efficacy was achieved for 3 p.m., 9 p.m., and the 3 a.m. groups. The median body weight and the leukocyte counts, however, suggest maximum toxic effects for the mice treated at 3 p.m. At this time point, the administered treatment was able to successfully regress the tumor but also had the maximum toxic effects to the normal tissue. The active phase of mice were therefore the best times to deliver the treatment. In the active phase, not much difference was observed in the tumor regression and toxicity for the treatments delivered at 9 p.m. or 3 a.m. The 9 a.m mice of the TPT and the XR group neither showed much tumor regression but also had some toxic effects. This is therefore concluded to be the worst time of treatment.

The previous study with p53+/+ tumors had shown that 3 a.m. time was the best to irradiate or administer TPT to the mice and 9 p.m the worst time of treatment delivery. The present study agrees with the previous findings for the X-radiation (4 Gy/fraction) treatment but the TPT (6 mg/kg) results were different.

Chapter 5

Conclusion

The circadian timed delivery of the treatments Topotecan and X-radiation did affect the tumor efficacy and the normal tissue toxicity. In the previous study, mice bearing HCT116 p53+/+ xenografts were treated with TPT or X-radiation at four different time points [14]. The treatment given during the late active period of mice i.e. 3 a.m. was found to be most effective. The amount of normal tissue toxicity was also lowest at this time. The XR results of present work agree with p53+/+ results suggesting that chronomodulation of XR treatment is potentially useful with optimal clinical therapeutic gain (i.e. increase in tumor kill accompanied by decrease in normal tissue toxicity) at late active phase (i.e. 3 a.m. in this study).

The TPT dose of 12 mg/kg was though considered high for this work because of p53-/- sensitivity to TPT [34], the results were similar to the p53 +/+ study. The treatment delivered at 3 a.m. achieved maximum tumor regression with minimum effect on the normal tissue. The 9 a.m. group, which is the early rest phase of mice, was the worst treatment time. In this group, no significant decrease in

the tumor volume was observed and the signs of toxicity were seen as per the body weight and the leukocyte count. The 3 p.m. group, which is the late rest phase of mice was effective in reducing tumor volumes but also had maximum toxic effects, making it unsuitable for effective treatment.

The chronomodulated treatment with drug TPT (6 mg/kg) presently remains inconclusive but could possibly suggest a concentration related effect. At this concentration, the maximum normal tissue toxicity as measured by body weight and leukocyte count did not agree with each other. Therefore, it was not possible to conclude from this work whether the p53 -/- tumors behaved similarly to the p53 +/+ tumors with TPT chronotherapy over a given range of TPT concentrations.

Overall, however, the chronomodulated treatments delivered to the human colorectal xenografts showed similar results for XR and TPT (12 mg/kg) treatment, irrespective of the p53 status. The only difference that was observed was in the sensitivity of p53. The abnormal p53 was more sensitive to the TPT treatments compared to the normal p53. The treatments in the active phase in general were more effective. The 9 p.m. group showed very similar results as the 3 a.m. group. The early rest phase, i.e. the 9 a.m. group, was not effective in reducing tumor volume with either TPT or X-Radiation.

Since normal tissues like gut and bone marrow are the dose limiting tissues, delivering treatments at the time when they are best tolerated gives a window of opportunity to increase the dose without increasing the side effects. Extrapolating the animal data (i.e. the best and worst treatment times) to humans requires more clinical trials because of the limited study parameters in the laboratory animals, but the chronomodulation study does provide an evidence of the advanced

therapeutic effects based on the widely researched circadian rhythms. The concept of chronomodulation, extrapolated from the mice to patients, was found valid in the study of phase III clinical trial for colorectal cancer metastases. But the long term survival rate still needs to be demonstrated [5].

5.1 Future studies

A possible explanation for the increase in the XR killing at 3 a.m. (late active phase) is due to increase in tumor blood flow which brings increase in the oxygen status. The latter brings about increased XR sensitivity due to well known oxygen effect [8]. The potential therapeutic gain of chronomodulated TPT drug could be due to the increase in drug delivery with increase in the tumor blood flow during the active period [32].

The circadian variation in the tumor blood flow to understand the underlying mechanisms of increased therapeutic effects during the late active period of mice can be studied using non-invasive MRI (Magnetic Resonance Imaging) technique. These experiments would suggest if there is any effect of tumor size on the circadian rhythm of the blood flow, or if relative differences are observed in the blood flow from the treatments (XR or TPT). Initial experiments using MRI sequences like HASTE (Half Fourier Acquisition Single shot Turbo spin Echo) sequence and EPI (Echo Planar Imaging) sequence were performed to check the feasibility of these experiments. Although more research needs to be done, the fast imaging MRI sequences can be used to image the mouse tumors without the use of anesthetics, which otherwise could alter the circadian patterns and their effect is difficult to reproduce in each mouse.

Experiments with lower TPT dose (e.g. 3 mg/kg) can be done to observe differences in the tumor volume regression and/or decrease in the body weight and leukocyte count.

The applicability of chronomodulated study of TPT and fractionated X-radiation can also be tested on other tumor types like OVCAR-3 xenografts (ovarian tumor cells) etc. This would help generalize the findings for potential clinical benefits to another human type where TPT is actually used clinically as a second line treatment [33].

Bibliography

- [1] Marie-Christine Mormont, Francis Lévi, “Cancer Chronotherapy: Principles, Applications, and Perspectives”. American Cancer Society, August 2003.
- [2] Francis Lévi, “From circadian rhythms to cancer chronotherapeutics”. *Chronobiology International*, 19: 1-19, 2002.
- [3] Tyvin A. Rich, Charles H. Shelton III, Alexander Kirichenko, and Martin Straume, “Chronomodulated chemotherapy and irradiation: An idea whose time has come?”. *Chronobiology International*, 19(1), 191-205, 2003.
- [4] W. J. M. Hrushesky, “Tumor chronobiology”. *Journal of controlled release*, 74: 27-30, 2001.
- [5] Francis Lévi, “Chronotherapeutics: The relevance of timing in cancer therapy”. *Cancer causes control*, 17: 611-621, 2006.
- [6] Sylvie Giacchetti, “Chronotherapy of colorectal cancer”. *Chronobiology International*, 19: 207-219, 2002.
- [7] M. C. Mormont and F. Lévi, “Circadian-system alterations during cancer processes: A review”. *Int. J. Cancer*, 70: 241-247, 1997.
- [8] Eric J. Hall, Amato J. Giaccia, “Radiobiology for the radiologist”. 6th Edition, Lippincott Williams & Wilkins, 2006.
- [9] Erhard Haus, “Chronobiology of the mammalian response to ionizing radiation potential applications in oncology”. *Chronobiology International*, 19: 77-100, 2002.
- [10] Leena Latonen, Marikki Laiho, “Cellular UV damage responses-Functions of tumor suppressor p53”. *Biochimica et Biophysica Acta*, 1755: 71-89, 2005.
- [11] Rosalyn D. Blumenthal, Chris Waskewich, David M. Goldenberg, Walter Lew, Christine Flefleh, and Jack Burton, “Chronotherapy and chronotoxicity of the cyclooxygenase-2 inhibitor, celecoxib, in athymic mice bearing hu-

man breast cancer xenografts". Clinical cancer research, Vol. 7, 3178-3185, October 2001.

- [12] URL: <http://www.graphpad.com/prism/prism.htm>
- [13] C.M. Ma, C. W. Coffey, L. A. DeWerd, C. Liu, R. Nath, S. M. Seltzer, J. P. Seuntjens, "AAPM protocol for 40-399 kV x-ray beam dosimetry in radiotherapy and radiobiology". Med. Phys. 28(6), June 2001.
- [14] Dana Mullins, "Chronomodulaion of topotecan and x-radiation therapy", Carleton University, 2004.
- [15] H. E. Johns, and J. R. Cunningham, "Physics of Radiology". Fourth Edition, Charles C. Thomas, 1983.
- [16] Dana Mullins, Denise Proulx, A Saoudi, Cheng Ng, "Chronomodulation of topotecan or X-radiation treatment increases treatment efficacwithout enhancing acute toxicity". Int. J. Radiation Oncology Biol. Phys., Vol. 62, No. 1: 230-237, 2005.
- [17] MacPherson M., "Clinical data for General Pantak Therapax HF300DT orthovoltage unit, Ottawa Regional Cancer Centre, Dept. of Medical Physics, Ottawa, ON", 1999.
- [18] Francis Lévi, "Circadian chronotherapy for human cancers: Review". The Lancet Oncology, Vol 2, May 2001.
- [19] Christopher G. Lis, James F. Grutsch, Patricia Wood, Mark You, Ivan Rich, and William J. M. Hrushesky, "Circadian timing in cancer treatment: The biological foundation for an integraive approach". Integrative cancer therapies, 2(2): 105-111, 2003.
- [20] Carla CB. Green, "Time for chronotherapy? Clock genes dictate sensitivity to cyclophosphamide". Proc. Natl. Acad. Sci., Vol 102, No. 10: 3529-3530, March 2005.
- [21] Samar A Jasser, David E. Blask, and George C. Brainard, "Light during darkness and cancer: relationships to circadian photoreception and tumor biology". Cancer causes control, 17: 515-523, 2006.
- [22] Eric J. Eide, and David M. Virshup, "Casein kinase I: Another cog in the circadian clockworks". Chronobiology International, 18: 389-398, 2001.
- [23] Maximilian Moser, and Karin Schaumberger, "Cancer and rhythm". Cancer causes control, 17: 483-487, 2006.

- [24] Teresa G. Granda, and Francis Lévi, "Tumor-based érythms of anticancer efficacy in experiment". *Chronobiology International*, 19: 21-41, 2002.
- [25] Francis Lévi, Rachid Zidani, and Jean-Louis Misset, "Randomised multi-centre trial of chronotherapy with oxaliplatin, fluoracil, and folinic acid in metastatic colorectal cancer ". *The Lancet*, 350: 681-686, 1997.
- [26] Satchidananda Panda, John B. Hogenesch, and Steve A. Kay, "Circadian rhythms from flies to humans". *Nature*, Vol. 417, May 2002.
- [27] Henry Q. Xiong, and Jaffer A. Ajani, "Treatment of colorectal cancer metastasis: The role of chemotherapy". *Cancer and metastasis reviews*, 23: 145-163, 2004.
- [28] Albert Goldbeter, and Daniel Claude, "Time-patterned drug administration: insights from a modelling approach". *Chronobiology International*, 19: 157-175, 2002.
- [29] Erhard Haus, "Chronobiology of the mammalian response to ionizing radiation potential applications in oncology". *Chronobiology International*, 19(1): 77-100, 2002.
- [30] Andrew Minchinton, and Ian F. Tannock, "Drug penetration in solid tumors". *Nature reviews*, Vol. 6: 583-592, August 2006.
- [31] Gang Liu, and Xinbin Chen, "Regulation of the p53 transcriptional activity". *Journal of cellular biochemistry*, 97: 448-458, 2006.
- [32] Dana Mullins, Denise Proulx, A Saoudi, and Cheng Ng, "Chronomodulation of Topotecan or X-radiation treatment increases treatment efficacy without enhancing acute toxicity". *Int. J. Radiation oncology Biol. Phys.*, Vol. 62, No. 1: 230-237, 2005.
- [33] Maurie Markman, "Topotecan as second-line therapy for ovarian cancer: dosage versus toxicity". *The Oncologist*, Vol. 10, No. 9: 695 - 697, October 2005.
- [34] Maja T. Tomicic, Markus Christmann, and Bernd Kaina, "Topotecan-triggered degradation of topoisomerase I is p53 dependent and impacts cell survival". *Cancer research*, Vol. 65, 8920-8926, October 2005.