Chronomodulation of Topotecan and X-Radiation Therapy

by

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Abstract

In recent years many advances have been made in the field of cancer therapeutics. Cure rates of most types of adult tumours, however, remain low. Chronomodulation (delivery of treatment at different times of the day or night) offers a method to improve treatment efficacy without increasing normal tissue toxicity. Topotecan (TPT) is a camptothecin (CPT) analog that is in active use in the treatment of ovarian cancer and small cell lung cancer. Ionizing radiation is used in the treatment of at least half of the cancer cases today. This work investigated whether chronomodulation of TPT treatments or x-radiation (XR) treatments could potentially lead to increased therapeutic index (i.e. increased treatment efficacy coupled with decreased normal tissue toxicity). Immune compromised, nude mice bearing human colorectal tumour xenografts were treated with either 12 mg/kg of TPT four times or 16 Gy of XR in 4 fractions of 4 Gy over two weeks. On the day of treatment, mice were treated at one of four times (9 a.m., 3 p.m., 9 p.m. or 3 a.m.) to evaluate the effect of chronomodulation on treatment efficacy and normal tissue toxicity. We found that for TPT or XR the group of mice treated at 3 a.m. showed the greatest amount of treatment efficacy when compared with the other three groups. Conversely, we found that the group of mice treated at 9 a.m. showed the greatest amount of acute toxicity, i.e., a significant decrease in the number of leukocytes in whole blood. This work suggests that chronomodulation of TPT or XR is potentially beneficial and may have clinical relevance.

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LIST OF ABBREVIATIONS

BSF	Backscatter Factor
CPT	Camptothecin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
HALO	Hours After Lights On
HVL	Half Value Layer
KVp	Peak Kilovoltage
MU	Monitor Unit
PBS	Phosphate Buffered Saline
PDD	Percentage Depth Dose
RDR	Relative Dose Rate
RT	Radiation Therapy
SSD	Source Surface Distance
Topo	Topoisomerase
TPT	Topotecan
XR	X-Radiation

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Chapter 1

Introduction

There are currently three major types of cancer treatment: surgery, radiation therapy (RT) and chemotherapy. For unresectable tumours, RT and chemotherapy are used. RT and chemotherapy are also used as adjuvants or adjuncts to surgery (i.e. to kill tumour cells that are not possible to remove, or that are inadvertently left after surgery). It has been estimated that at least 50% of cancer patients undergo RT at some point. Inability to achieve local tumour control is a major cause of cancer treatment failure. Clinically, local tumour control cannot always be achieved due to the inability of the patient's tissue to withstand the dosing levels required to achieve treatment success. Since we are still unable to achieve this goal of success in many types of adult tumours, it is necessary to develop new ways of treating cancer more effectively. One such idea to improve the efficacy of certain types of chemotherapy and RT treatments that is now being considered is chronomodulation. Chronomodulation refers to delivering treatments at different times of the day or night. Chronotherapy of cancer, therefore, employs chronomodulation techniques either to achieve increased treatment efficacy (with decreased normal tissue toxicity) or to improve patient quality of life by reducing normal tissue toxicity.

1.1 The Circadian Rhythm

It has been observed that plants and animals experience daily oscillations in physiological as well as behavioural processes, called the circadian rhythm. (Fu and Lee, 2003). Circadian rhythms exist in all organisms and at all levels of organization within an organism. Blood flow, cell proliferation, metabolic activity and hormonal levels (Fu and Lee, 2003) are just a few of the processes that vary throughout the day due to the circadian rhythm. In living species (including humans), the circadian rhythm has a genetic origin (Fu and Lee, 2003) although the diurnal alteration of light and dark plays an important role in the synchronization of the daily rhythm (Arendt, 1998). It has been shown that a disruption in the normal circadian rhythm can have adverse consequences (e.g. coronary heart attacks, depression and cancer) (Touitou and Haus, 1992; Mormont and Levi, 1997; Roenneberg and Lucas, 2002). Tumour tissues also exhibit a circadian rhythm although often with different phase (and amplitude) than those found in normal tissues (Smaaland *et al.*, 1995). A different circadian rhythm between tumour and normal tissues may provide a window of opportunity to improve cancer treatment by allowing treatment doses to be escalated at a time of increased tumour tissue sensitivity while the normal tissue is relatively insensitive. Alternatively, in the case of combined treatment with more than one agent, (e.g. x-radiation with drug), chronomodulation principles can be applied to time the delivery of the second agent at a time of increased tumour sensitivity and/or decreased normal tissue sensitivity following treatment with the first agent. This is the basic principle underlying the chronotherapy of cancer.

1.2 Chronotherapy of Cancer

Chronotherapy means varying the time of delivery to achieve optimum results. Chronotherapy utilizes naturally occurring biological rhythms to optimize treatment efficacy and host tolerance of a treatment. The concepts of chronobiology have been investigated to improve the therapeutic index of cancer drugs. These studies have shown that most drugs demonstrate temporal pharmacokinetic variations depending on the time of administration (Arendt, 1998). Dosing time for many drugs markedly affects the safely achievable dose and most normal tissues are more or less sensitive to drug effects at certain times of the day.

There is much evidence to support the relevance of chronotherapy. When given to mice, the toxicities and anti-tumour activities of approximately 30 chemotherapeutic agents can vary by more than two-fold according to dosing time (Misset and Levi, 2000; Mormont and Levi, 2003). Bone marrow and gastrointestinal tissues are common dose-limiting tissues for many chemotherapeutic agents (Granda *et al.*, 2002). A circadian variation in the cell cycle (Figure 1.1) of the cells in these tissues could contribute to the effectiveness of chronotherapy. There is a peak in the deoxyribonucleic acid (DNA) synthesis and mitotic activity that could allow for dose escalation at the appropriate time. In addition, the circadian rhythm of tumour and normal tissue blood flow (Hori *et al.*, 1996; Blumenthal *et al.*, 2000) can markedly affect the delivery of the drug to these tissues. Clinically, the paradigm of chronotherapy has been most widely examined and validated in human metastatic colorectal cancer (Mormont and Levi, 2002). For tumour-bearing animals, it has been shown that the time of least normal tissue toxicity

corresponds to the time of best tumour control (Levi, 1997; Peters et al., 1987; Sothorn et al., 1989). As for human patients, in clinical trials involving more than 2000 patients treated with a combination of 5-FU, folinic acid, and oxaliplatin, the chronomodulated arm was associated with a significantly improved therapeutic index (i.e. better objective response, less peripheral neuropathy, longer median time to failure) (Levi *et al.*, 1997; Levi *et al.*, 1994).

Figure 1.1 Schematic representation of the cell cycle. 'G1' stands for 'Gap 1' and 'G2' stands for 'Gap 2' so named because they were (incorrectly) thought to represent periods between S and M when nothing visually interesting happened inside the cell.

1.3 Camptothecin and its Analogs

Camptothecins (CPT) are a well-known class of compounds that target DNA topoisomerase (topo) I, an enzyme that is important for numerous cellular activities including DNA replication and transcription. CPT analogs (e.g. irinotecan, CPT-11; topotecan, TPT) are already being used to treat certain human malignancies (US National Cancer Institute, 2003). TPT is used for treating ovarian cancer (SmithKline Beecham Inc., 1997) and is currently in clinical trials for various other tumour sites (e.g. acute myeloid leukemia, small cell lung cancer) (US National Cancer Institute, 2003). The mechanism of action of CPT and its analogs involves the formation of cleavable complexes that interfere with topo I-regulated breakage rejoining of DNA resulting in cell death (SmithKline Beecham Inc., 1997). Consequently, CPT analogs are effective S-Phase targeting agents, i.e. they show preferential toxicity for S-phase cells (Del Bino et al., 1992). Actively proliferating tumour cells are very sensitive to S-phase targeting agents.

Although somewhat surprising initially, it is now known that normal cells undergo an orderly progression through the cell cycle with the majority of cells in a specific organ entering the S-phase at a particular time of day. Interestingly, chronomodulation may be especially important for S-phase targeting agents because of the asynchrony in circadian rhythm of DNA synthesis between normal and tumour tissues (Sheving et al., 1995). For example, peak administration of 5-FU, which also targets S-phase cells, to patients at 12 a.m. (i.e. human rest period) is better tolerated and more efficacious than standard 5-FU therapy (De et al., 1996). In addition, CPT-11 (Ohdo et

al., 1997; Granda et al., 2002) treatments given during the active phase of mice are associated with increased acute toxicity (i.e. body weight loss, intestinal and haematological toxicities). In the case of 9-AC, acute normal tissue toxicity was the greatest at 2 a.m. (20 HALO) for mice given both single and multiple doses (Kirenchenko, 1999). The dose of 9-AC given during the rest phase of mice could be safely escalated by 33% (Kirenchenko, 1999). To date, there is no data to suggest whether the therapeutic index of TPT could potentially be enhanced by chronomodulation. The fact that TPT is a known S-Phase targeting agent and no that previous data exists made it an excellent choice for this study.

1.4 Chronomodulation of X-Radiation

Humans are constantly exposed to radiation from a variety of sources both natural and man-made throughout their lives (Haus, 2002). The main sources of natural radiation are cosmic radiation and radiation from radioactive elements found in the Earth's crust (Haus, 2002). Exposure to manufactured radiation sources occurs mainly because of medical diagnosis and/or treatment (Haus, 2002).

Ionizing radiation is currently used to treat a large percentage of all patients with cancer (Parker and Withers, 2001). As is the case with chemotherapy, the effectiveness of radiation therapy is often limited by the ability of the patient's normal tissues to withstand the dose required to destroy the tumour.

The target of greatest radiobiological importance is the DNA of cells (Haus, 2002; Hall, 2000). There are two different ways that radiation can damage DNA. The first is by a direct "hit" or absorption of an electron by DNA and the second is by indirect damage caused by free radicals which have been produced in the cell by the radiation (Puck, 1972; Puck and Marcus, 1956; Sinclair, 1968). Figure 1.2 illustrates the two different types of DNA damage. For x-radiation, indirect damage is relatively more important.

Figure 1.2 The 2 modes of interaction of radiation with DNA, direct and indirect.

When various cell populations that contain cells in a synchronized stage of the cell cycle (Figure 1.1) are irradiated, the cells in G2 and M show the highest radiosensitivity, the cells in G1 show less sensitivity and those towards the end of the S-Phase are the least sensitive (Haus, 2002). For mammals, the most rapidly dividing cells in the body are found in the bone marrow and the crypt-epithelium of the intestine, and they are the most radiosensitive (Haus, 2002). These tissues are dose limiting when planning radiation treatments especially when they are in the field of irradiation (Haus, 2002). When they are not in the field of irradiation a higher dose can be used and the normal tissue toxicity is not as severe.

It has been reported that for mice irradiated with both 800 R and 900 R the total body radiosensitivity showed a peak at 19 hours after light on (HALO) (late active phase) (Pizzarello et al., 1963; Pizzarello et al., 1964). It was later shown that this peak corresponds with the peak of mitosis in bone marrow with the S-Phase peak in bone marrow occurring at 15 HALO (Pizzarello and Witcofski, 1970). For rats with and without Pliss lymphosarcoma, it has been shown that the circadian rhythm of mitotic activity of bone marrow has a peak occurring shortly after the onset of dark (Blank, 1995). It has also been shown that the circadian rhythm of mitotic activity of the tumour tissues has a much smaller amplitude and a different peak that occurs late in the dark span (Blank, 1995).

Due to the circadian variation in mitotic activity in bone marrow, the dose limiting tissue for many drugs and radiation treatment, chronomodulation may help to improve the therapeutic index of radiation treatment.

1.5 Goals and Hypotheses

The goal of this research was to determine the treatment efficacy and normal tissue toxicity associated with TPT or fractionated X-radiation treatments. These treatments were given either during the active (dark-phase), or rest (light phase), period of mice bearing human colorectal cancer tumours to establish the role of chronomodulation.

The two hypotheses being tested were:

1. TPT treatments given during the rest phase will be associated with less normal tissue toxicity and increased treatment efficacy and will result in an enhanced therapeutic gain.
2. X-radiation treatments given during the active phase will be associated with less normal tissue toxicity and increased treatment efficacy and will result in an enhanced therapeutic gain.

The efficacy of both the TPT and radiation treatments was assessed from the ability of the treatment to shrink the tumour. Normal tissue toxicity was assessed from loss of body weight and from changes in the number of leukocytes present in whole blood.

Chapter 2

Materials and Methods

2.1 Mice

To study the effects of chronomodulation it was necessary to inject the drug as well as irradiate the mice at various times of the day and night. Administering treatments to the mice during the night is not practical for various reasons (e.g. inconvenience, staff unavailability); therefore, half of the mice were light shifted in order to achieve the effect of treating during the night. The mice were given treatment (either TPT or X-radiation) at 9 a.m. and 3 p.m. resulting in four different effective times for treatment (i.e. 9 a.m. (3 HALO), 3 p.m. (9 HALO), 9 p.m. (15 HALO) and 3 a.m. (21 HALO)). It is important to note that mice, like other rodents, are nocturnal. Mice are active during the night (i.e. when the lights are off) and rest during the day (i.e. when the lights are on). Therefore, for mice 9 a.m. is the early rest phase, 3 p.m. is the late rest phase, 9 p.m. is the early active phase and 3 a.m. is the late active phase.

Upon arrival the mice were immediately separated into two groups, those that would maintain a normal light cycle (lights on at 6 a.m., lights off at 6 p.m.) and those that were to be light shifted (lights on at 6 p.m., lights off at 6 a.m.). The light shifted group was given two weeks to adjust to the change in light cycle with the change being introduced gradually over the first four days (day 1: lights off 3 p.m., lights on 3 a.m.,

day 2: lights off 12 noon, lights on 12 a.m., day 3: lights off 9 a.m., lights on 9 p.m. and day 4: lights off 6 a.m., lights on 6 p.m.). For a given experiment control mice were given a sham injection, while those used for experiments were given an injection of topotecan or irradiated at a given time point, either 9 a.m. or 3 p.m.

The mice used in this study were all CD-1 nude females (Figure 2.1), obtained from Charles River Laboratories, Wilmington, MA. Nude mice are immune compromised. To avoid any unnecessary illness among the mice all housing materials were autoclaved prior to use and all handling was done in a hood while wearing sterile gowns and gloves, face masks, hair bonnets and shoe covers. All experiments were approved by the animal care facility at the University of Ottawa and met with their ethical guidelines. Mice were housed two per cage and were given sterilized food and acidic water *ad libitum*. The mice in the light shifted group were all two weeks younger at the start of the shifting process than the mice in the normal group, to account for the time required for light shifting, with all the mice beginning to receive treatment at ten weeks of age.

Figure 2.1 A nude CD-1 female mouse with a tumour on the flank.

2.2 Cell Line

HCT116 human colorectal cells obtained from Dr. B Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD) were used for this study. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco). The cells were incubated at 37 °C in 5% CO₂ and 95% air.

Cells were grown on 30 cm dishes until confluent. To remove the cells from their dishes the media was removed and the cells were treated with 2 mL of the proteolytic enzyme trypsin (0.2 % w/v trypsin in citrate saline (Gibco)) for 5 min at 37 °C to loosen them from the surface of the dish. Trypsin action was stopped by adding DMEM with 10% FBS. Cells were then collected, centrifuged and washed with serum-free DMEM. A sample of cells was counted using a Bright Line haemocytometer and the cells were then resuspended in serum-free media at a concentration of 2.5×10^7 cells/mL. To grow the tumours each mouse was injected sub-cutaneously with 200 µL of the cell suspension for 5×10^6 cells. The tumours were allowed to form for 12 days prior to the first treatment. These cells do not metastasize to other organs.

2.3 Drug Preparation

The drug being studied was Topotecan, sold as Hycamtin[®] by SmithKline Beecham, Oakville, ON. The dose used for injection was 12 mg/kg. The drug was obtained in a vial containing 79 mg of Hycamtin[®], which contained 4 mg of active drug. It was used as a 3 mg/ml solution of active drug, dissolved in phosphate buffered saline (PBS). For example, to make 1 mL of the 3 mg/mL solution to be injected:

$$\frac{3 (79)}{4} = 59.25 \text{ mg of the powder as sold was dissolved in 1 mL of PBS.}$$

Injections were given four times, twice a week for the first two weeks. The injection days were recorded as days 0, 4, 7 and 10 of the experiment. The drug was injected intra-peritoneally under aseptic conditions.

2.4 Calibration of the Irradiator

On the basis of initial experiments, it was decided to deliver a dose of 4 Gy to each mouse 4 times corresponding to the same days as drug delivery for a total of 16 Gy during the experiment. Due to the various tumour shapes, it was also decided to calculate the dose based on delivery to the surface of the tumour. The x-ray unit (irradiator) used to irradiate the mice was a Pantek Bipolar Series model HF230 (referred to herein as the research irradiator). The clinical irradiator used as part of the calibration was a General Pantak HF300DT Orthovoltage Unit (referred to herein as the clinical irradiator). The research irradiator was normally used to irradiate cells *in vitro* at a potential of 250 kVp. The geometry of the research irradiator was such that the beam originated from above the mouse. Since the tumours were located in the backs of the mice, 250 kVp was deemed too high for this situation. It was instead decided to use an operating potential of 100 kVp to minimize damage to the normal tissues (in particular, gastrointestinal system) directly below the tumour. Because of the method used to perform the calibration 100 kVp was the lowest voltage that could be used.

A plot of percentage depth dose vs. depth for the clinical irradiator with an 8 cm cone, at 100 kVp and with a 1.65 mm Al filter is shown in Figure 2.2. This figure indicates that at a depth of 4 cm, which would be the region of normal tissue damage for the mice, the dose has already been reduced to approximately 35%. This was deemed acceptable and 100 kVp was used to irradiate the mice.

Figure 2.2 Percentage depth dose vs. depth for the clinical irradiator with an 8 cm cone, at 100 kVp and with a 1.65 mm Al filter, data from (MacPherson, 1999).

To determine the length of time needed to deliver a dose of 4 Gy to the surface of the tumour the relative dose rate (RDR) for the clinical irradiator was used along with readings obtained from both the clinical irradiator and the research irradiator. It was necessary to use the clinical irradiator because the RDR of the clinical irradiator was readily available; this information was not available for the research irradiator. The clinical irradiator was similar to the one used for this experiment except that the x-ray tube was not contained inside a steel box which allows for the treatment of patients. The x-ray tube used to irradiate the mice was enclosed in a steel cabinet for radiation protection of laboratory staff.

To ensure that the two irradiators were in fact similar enough to allow the same data to be used for both, the half value layer (HVL) of the research irradiator was determined. For the clinical irradiator at a setting of 100 kVp, with a 1.65 mm Al filter the HVL is 2.57 mm Al (MacPherson, 1999). To determine the HVL of the research irradiator under the same conditions an Al filter, approximately 1.6 mm in thickness was constructed for the research irradiator and was put in place of the flattening filter normally used when irradiating cells.

All data were acquired using the same ionization chamber and electrometer. The ionization chamber used was a Farmer Ionization Chamber, Model NE 2571, 0.6 cc. The electrometer used was a 2570 Farmer Dosimeter from NE Technology. With the 1.6 mm Al filter in place data were collected using the set-up shown in Figure 2.3. The HVL was found by measuring the exposure rate with a variety of aluminium sheets of known thickness placed in the beam (Johns and Cunningham, 1983).

Figure 2.3 Set-up used to measure the HVL of the research irradiator. The ionization chamber was sitting on the lid of the foam box (which was inverted), ~50 cm from the bottom of the irradiator cabinet, to avoid backscatter.

The data obtained are given in Table 2.1. A plot of the data used to determine the HVL of the clinical irradiator is shown in Figure 2.4. It should be noted that the 1.6 mm Al filter was in place during all of these measurements and the thickness of Al indicated was in addition to the filter present. Using the equation found from Figure 2.4 the HVL was determined to be 2.38 mm Al. The value of the HVL for the clinical irradiator is 2.57 mm Al (MacPherson, 1999). This was a difference of 7.4% and was deemed appropriate since it was within 10%.

Table 2.1 Data obtained for the measurement of the HVL of the research irradiator

mm of Al	Reading 1 (R)	Reading 2 (R)	Reading 3 (R)	X (R)	X/X ₀	Sigma
0	92.27	92.32	92.32	92.30	1	0.0136
0.51	75.85	75.92	75.92	75.90	0.8223	0.0166
1.02	64.45	64.42	64.47	64.45	0.6982	0.0128
1.53	56.05	56.10	56.10	56.08	0.6076	0.0136
2.03	49.70	49.70	49.72	49.71	0.5385	0.0104
2.54	44.37	44.40	44.45	44.41	0.4811	0.0166
3.05	40.15	40.15	40.17	40.16	0.4351	0.0104

Figure 2.4 Plot of the data used to find the HVL of the research irradiator.

The source surface distance (SSD) was set at 30 cm, with the aperture of the lead being a circle with a 2 cm diameter, located 7.5 cm from the origin of the x-rays. With these conditions, it was determined that the field size at the surface of the tumour would be a circle with an 8 cm diameter. To ensure that the diameter of the field was in fact 8 cm a film was exposed which was placed at a distance of 30 cm from the source. An optical density trace of the film, Figure 2.5, showed that the field size was in fact an 8 cm circle.

Figure 2.5 Spot-size of the research irradiator at 30 cm SSD with aperture and filter in place. This is an optical density measurement of the exposed film with a picture of the film inset. The data were within 5 % of the ideal case and this was deemed acceptable.

To find a calibration factor between the readings obtained from the electrometer and the dose at the surface in cGy the RDR of the clinical irradiator was used along with a series of readings for a given monitor unit (MU). The RDR of the clinical irradiator is:

$$\text{RDR} = 2.973 \text{ cGy/MU}$$

It was also known that the clinical irradiator delivers 130.4 MU/min or 2.17 MU/s. Using this in combination with the RDR gives a dose rate of 6.461 cGy s^{-1} . The clinical irradiator was set to deliver a dose of 40 MU and a series of four readings were taken with the SSD set at 30 cm and a cone in place to ensure a circle of 8 cm diameter at the detector, the same situation as the research irradiator. The readings obtained were 100.22, 100.20, 99.90, and 100.10 with the average being 100.11 R.

Knowing that 40 MU corresponds to a time of 18.40 s a calibration factor between time and electrometer reading could be found

$$\text{Calibration Factor} = \frac{6.461 \text{ cGy s}^{-1} 18.40 \text{ s}}{100.11 \text{ R}}$$

$$\text{Calibration Factor} = 1.188 \text{ cGy / R}$$

Readings were taken using the research irradiator and those reading were converted to dose using the calibration factor found from the clinical data. The readings obtained are given in Table 2.2. A plot was then made using the data for time and the dose at surface, the equation of which was used to determine the time required for a 4 Gy dose.

Table 2.2 Time settings and their corresponding readings for the research irradiator. The readings have been converted to doses using the calibration factor, 1.188 cGy/R.

Time (s)	Reading (R)	Dose at surface (cGy)
22	45.50	54.05
33	71.14	84.66
44	97.20	115.5
45	99.23	117.9
60	134.31	159.8

Figure 2.6 Time vs. dose at surface for the research irradiator. This is a plot of the data of Table 2.2. The dose delivered is proportional to the time of irradiation and the equation found is valid for the dose of interest. Error bars are too small to be visible.

Using the equation found from Figure 2.6, the time required for irradiation was determined to be:

$$\text{Time (s)} = \frac{0.3593 (\text{Dose (cGy)}) + 2.5755}{\frac{\text{BSF (cut out)}}{\text{BSF (8 cm)}}}$$

For 400 cGy, which was the dose of interest in our experiment, this becomes:

$$\text{Time (s)} = \frac{0.3593 (400) + 2.5755}{\frac{\text{BSF (cut out)}}{\text{BSF (8 cm)}}}$$

$$\text{Time (s)} = \frac{146.30 \text{ s}}{\frac{\text{BSF (cut out)}}{\text{BSF (8 cm)}}}$$

Thus, for an open field to deliver a dose of 4 Gy to the surface one would need to irradiate for 146 s. This time was altered based on the size of the cut out in the lead shielding.

Using a HVL of 2.4 mm of Al the backscatter factors (BSF) needed to be determined. Using the AAPM protocol (Ma et al., 2001) water kerma based backscatter factors for a water phantom as a function of field diameter and HVL were obtained for a SSD of 30 cm (Ma et al., 2001) and are given in Table 2.3. Data for 2.4 mm Al were interpolated, as was the data for a diameter of 8 cm since those were the dimensions of interest and they were not listed in the protocol.

Table 2.3 Water kerma based backscatter factors.

	HVL (mm Al)			
Diameter (cm)	2.0	2.4	3.0	BSF (cut out)/BSF (cone)
1	1.058	1.060	1.063	0.857
2	1.108	1.113	1.120	0.900
3	1.140	1.150	1.164	0.930
5	1.182	1.198	1.221	0.969
8	1.216	1.236	1.267	1.000
10	1.238	1.262	1.298	1.021

The BSF ratio was then plotted (Figure 2.7) and an equation of best fit was found. The equation found was used to determine the value used for the BSF for various diameters of cutouts. Table 2.4 lists the cut out sizes that were used along with the length of time for irradiation for each of these cut outs.

Table 2.4 Cut out diameters and their corresponding irradiation times.

Diameter (cm)	Time (s)
0.63	173
0.70	173
0.78	172
0.94	170
1.09	169
1.25	167

Figure 2.7 BSF (cutout)/BSF (cone) vs. diameter for an 8 cm diameter cone. Error bars are too small to be visible.

2.5 Construction of Mouse Restraint

Because anaesthetics could affect blood flow (and this effect could be difficult to reproduce from mouse to mouse) and thus alter TPT or XR response, it was desired to restrain, rather than anaesthetize the mice during the XR treatment. There was no device available for such a purpose at our centre so it became necessary to construct an appropriate restraint. The main component of the restraint was a 50 mL falcon tube. The diameter of this type of tube was such that the mice fit snugly inside with little room to move. A slit was cut in the tube to allow the tumour to protrude; there were a variety of different slit lengths and widths used to accommodate the various tumour sizes. There was an opening at the front of the tube to allow the mouse to breath. The mouse was held in place with a plug that had a hollow center to allow for the tail of the mouse to protrude. Two Teflon[®] rings were used to hold the tube tight from the outside to ensure that the mouse would not be able to become free during irradiation. The outsides of the rings were covered with the soft half of Velcro[®] to allow for easy attachment to the lead shielding. A mouse inside the irradiation restraint is shown in Figure 2.8. One can clearly see the tumour protruding from the slit in the tube and the tail of the mouse protruding from the end of the plug. The Teflon[®] rings were in place and prevented the mouse from loosening the plug.

Figure 2.8 A mouse bearing a tumour inside the irradiation restraint with its tumour clearly visible and protruding from the restraint.

For the clinical irradiator with the 1.65 mm Al filter in place at a potential of 100 kVp the transmission through 1.0 mm of lead was 0.3% (MacPherson, 1999). A lead shield of 2.0 mm thickness was used to ensure that no irradiation damage would occur other than through the cut out. There were 6 different lead shields used all with different cut out sizes. The shields were all 20 x 20 cm, so there was no risk of the 8 cm spot size exceeding the shielded area. The cut out sizes were listed previously in Table 2.4. The rough side of the Velcro® was attached to the bottom of the each of the lead shields to allow the restraint to be attached using the other half of the Velcro®, attached to the rings. Care was taken to ensure that the tumour was aligned with the cut out in the shield. With the mouse in the restraint and the lead shield properly attached the restraint and shield were placed in a sterile cage and then the mouse was irradiated for the appropriate amount of time. It is important to note that all parts of the restraint were sterilized prior to use and that the mouse was put in the restraint under aseptic conditions. Figure 2.9 shows a mouse in a restraint with one of the lead shields attached. Figure 2.10 shows a top view of the lead shield with the mouse's tumour visible through the cut out.

Figure 2.9 A mouse inside the restraint, which was then attached to the lead shield.

Figure 2.10 A top view of one of the lead shields with a restrained mouse in place for irradiation with the tumour visible through the cutout in the lead.

2.6 Measurement of Tumours

To determine the effectiveness of the treatment, the tumours were measured three times a week beginning on the first day of treatment for a total of four weeks. All measurements were done using a caliper (Figure 2.10) and three orthogonal dimensions of each tumour were recorded: the length, width and depth. The formula used to compute the volume was:

$$V = \frac{\pi}{6} l w d ,$$

with l being the long axis of the tumour, w being the short axis of the tumour and d being the height of the tumour above the surface of the mouse. This formula assumes that the tumours had an ellipsoidal shape and reduces to the volume of a sphere for $l = w = d = 2r$.

As per the guidelines of the animal care facility, a mouse was removed from the experiment if the tumour volume exceeded 20% of the mouse's body weight (i.e. if any one dimension was greater than 16 mm) or the tumour showed any signs of ulceration. If this happened prior to the end of the treatment phase (i.e. during the first 2 weeks) the mouse was not considered as part of the group. The caliper was sterilized prior to each day of use and the tumour measurements were all done under aseptic conditions to avoid illness among the mice.

Figure 2.11 Caliper used to measure tumour dimensions.

2.7 Measurement of Normal Tissue Effects

Two different methods were used to measure the normal tissue effects in the mice. The first method was the measurement of body weight. Mice were weighed twice a week on the same day as the treatments were administered and continuing for two more weeks after treatment, for eight measurements throughout the duration of the experiment.

The second method used to determine the normal tissue effects was the number of leukocytes present in the blood. Blood samples were obtained from each mouse once a week throughout the experiment using the UNOPETTE[®] Microcollection System (Becton, Dickinson & Co., Canada, Ltd., Mississauga, ON). The number of leukocytes in one mm³ of blood was calculated by counting a representative sample using a Bright Line haemocytometer.

2.8 Statistical Analysis

Since we were dealing with living animals, some mice had to be euthanised because of tumours that were too large or because their tumours had become ulcerated as stated previously. Occasionally the tumours failed to grow and therefore no data could be obtained from a particular mouse. Because of these and other problems, we were left with groups that did not contain the same number of mice and with decreasing mouse numbers throughout the duration of the experiment. Because of biological variation from one mouse to another the distribution of tumour volumes at a given time for a given group did not follow a normal distribution. This was especially true after treatment as some mice had only very tiny tumours whereas others had to be euthanised early because of extremely large tumours.

Generally, a statistical test requires an assumption about the distribution of the population from which the samples are drawn (Spiegel, 1994). In reality, there are cases in which such assumptions may not be warranted, as is often the case with many studies involving living organisms. To accommodate this type of data various tests and methods have been devised that are independent of population distributions and associated parameters. These are called non-parametric tests.

The Mann-Whitney test, also called the rank sum test, is a nonparametric test that compares two unpaired groups. To perform the Mann-Whitney test one must first sort all the sample values from both groups in a list from the smallest to largest and assign each value a rank. If two values are the same, then they both get the average of the two ranks for which they tie. The smallest number gets a rank of 1. The largest number gets a rank of N , where N is the total number of values in the two groups combined. Next, the sum

of the ranks for each of the samples must be found. The sample sizes are denoted N_1 and N_2 with R_1 and R_2 being the respective rank sums. A significant difference between R_1 and R_2 implies a significant difference between the samples. P values were obtained using the program GraphPad Prism (GraphPad Software, Inc., San Diego, CA). If the P value was found to be less than 0.05, the samples were deemed significantly different. If the P value was found to be large (i.e. greater than 0.05), it was concluded that the medians were not significantly different. In case of small samples, the Mann-Whitney test had little power. If the total sample size was seven or less, the Mann-Whitney test always gave a P value greater than 0.05 no matter how much the groups differed, therefore we were careful to ensure that there was a total of at least 7 samples (treated plus its matched control) for each group tested.

Chapter 3

Results

The Mann Whitney test was used to analyse all the data. Those mice treated with TPT were tested against their matched control and the same was done with those mice that were treated with XR. Time-matched control mice were sham injected with PBS. All of the results are presented in two different ways. First, they are presented in tabular form with the number of samples, medians, P values and an indication of whether there was a statistically significant difference between the treated group and its matched control. This is done for each time point (9 a.m., 3 p.m., 9 p.m. and 3 a.m.), each type of treatment (TPT and XR), and each parameter evaluated (tumour volumes, body weights and leukocyte counts). Second, the results are presented graphically with the median values for each parameter plotted, one plot for each time for each parameter.

3.1 Tumour Volumes

Tumour volume measurements were used to assess the efficacy of the treatment at controlling the disease. A significant difference between a treated group and its matched control is considered as treatment success if the treated group has a smaller tumour volume than its matched control. At 9 a.m., there was only one point, on day 18, when the group treated with topotecan differed significantly from the control group, shown in Table 3.1. Table 3.2 shows that on day 4 there was a difference between the group treated with TPT at 3 p.m. versus the 3 p.m. control group, however, the control group

has a smaller tumour volume, so this was not considered as treatment success. At 9 p.m., the TPT treatment was effective on days 16, 18 and 21 as shown in Table 3.3 and on each of these days the treated group had a smaller median than the control so the treatment was deemed as successful on those days. Among the groups treated with TPT the 3 a.m. group showed the most days where there was a statistically significant difference between the treated and control groups. The treated group was statistically smaller on days 9, 14, 16, 18, 21, 23 and 25; the data for this group are shown in Table 3.4.

Table 3.1 Data and statistical analysis results for tumour volumes of mice treated with 12 mg/kg of TPT at 9 a.m. vs. the 9 a.m. control group.

Day	Number of mice		Medians (mm ³)		P value	P < 0.05?
	TPT	Control	TPT	Control		
0	14	14	88.0	54.6	0.1479	No
2	14	14	128	93.6	0.1544	No
4	14	14	120	129	0.4082	No
7	14	14	193	236	0.6961	No
9	14	14	179	347	0.2064	No
11	13	12	211	394	0.1826	No
14	13	9	223	558	0.1419	No
16	11	6	211	343	0.1451	No
18	8	5	155	374	0.0186	Yes
21	8	3	153	299	0.3758	No
23	8	3	243	426	0.4970	No
25	7	3	187	378	0.1167	No

Table 3.2 Data and statistical analysis results for tumour volumes of mice treated with 12 mg/kg of TPT at 3 p.m. vs. the 3 p.m. control group.

Day	Number of mice		Medians (mm ³)		P value	P < 0.05?
	TPT	Control	TPT	Control		
0	15	12	93.6	58.3	0.1571	No
2	15	12	105	76.1	0.1021	No
4	15	12	140	83.2	0.0040	Yes
7	15	12	208	154	0.0604	No
9	15	12	257	222	0.3291	No
11	15	11	311	243	0.3370	No
14	15	11	328	286	0.2327	No
16	13	7	218	252	0.8741	No
18	12	5	220	275	0.9580	No
21	11	3	256	156	0.5334	No
23	7	3	312	232	0.9999	No
25	5	3	253	243	0.9999	No

Table 3.3 Data and statistical analysis results for tumour volumes of mice treated with 12 mg/kg of TPT at 9 p.m. vs. the 9 p.m. control group.

Day	Number of mice		Medians (mm ³)		P value	P < 0.05?
	TPT	Control	TPT	Control		
0	13	13	99.5	61.4	0.4418	No
2	13	13	137	72.8	0.0613	No
4	13	13	150	109	0.1742	No
7	13	13	133	155	0.5215	No
9	13	13	139	164	0.6081	No
11	13	12	133	190	0.0685	No
14	13	11	154	283	0.0597	No
16	11	11	115	483	0.0031	Yes
18	11	10	140	417	0.0008	Yes
21	11	8	200	683	0.0026	Yes
23	11	7	250	712	0.1031	No
25	9	5	250	314	0.8981	No

Table 3.4 Data and statistical analysis results for tumour volumes of mice treated with 12 mg/kg of TPT at 3 a.m. vs. the 3 a.m. control group.

Day	Number of mice		Medians (mm ³)		P value	P < 0.05?
	TPT	Control	TPT	Control		
0	13	11	120	98.3	0.068	No
2	13	11	164	117	0.2129	No
4	13	11	166	147	0.7943	No
7	13	11	172	218	0.2129	No
9	12	11	167	325	0.0051	Yes
11	12	9	180	304	0.1179	No
14	11	5	147	343	0.0127	Yes
16	11	5	125	329	0.0092	Yes
18	11	4	150	506	0.005	Yes
21	11	4	141	613	0.0075	Yes
23	10	3	181	811	0.014	Yes
25	9	3	187	874	0.0182	Yes

Tables 3.5 – 3.8 contain the tumour volume data and analysis results for those mice that were treated with x-radiation. The data in Table 3.5 are for those mice that were treated with x-radiation at 9 a.m. The Mann-Whitney test revealed that there were 3 days when there was a significant difference between the treated group and the control group, namely, days 9, 16, and 18. On all three of these days the tumours in the treated group were significantly smaller than those in the control group. Table 3.6 contains the data for those mice that were treated with x-radiation at 3 p.m. There was no significant difference between the treated group and the control group at any time. Table 3.7 contains the data for those mice that were treated with x-radiation at 9 p.m. There were three days when the tumour volumes of the treated group were significantly smaller than those of the control group, namely, days 16, 18 and 21. Table 3.8 contains the data for those mice treated with x-radiation at 3 a.m. On 10 of the 12 days when tumours

volumes were measured, the treated group was significantly smaller than the matched control. The only 2 days when there was no significant difference between the treated and control groups were days 0 and 4, which occurred early in the experiment.

Table 3.5 Data and statistical analysis results for tumour volumes of mice treated with 4 fractions of 4 Gy of XR at 9 a.m. vs. the 9 a.m. control group.

Day	Number of mice		Medians (mm ³)		P value	P < 0.05?
	XR	Control	XR	Control		
0	8	14	66.5	54.6	0.9728	No
2	8	14	85	93.6	0.5618	No
4	8	14	91.5	129	0.6573	No
7	8	14	169	236	0.3224	No
9	8	14	163	347	0.0186	Yes
11	8	12	194	394	0.0826	No
14	7	9	219	558	0.0229	No
16	7	6	172	342	0.0513	Yes
18	7	5	240	374	0.0177	Yes
21	6	3	143	299	0.1667	No
23	6	3	199	426	0.1667	No
25	6	3	175	378	0.0952	No

Table 3.6 Data and statistical analysis results for tumour volumes of mice treated with 4 fractions of 4 Gy of XR at 3 p.m. vs. the 3 p.m. control group.

Day	Number of mice		Medians (mm ³)		P value	P < 0.05?
	XR	Control	XR	Control		
0	10	12	82.1	58.3	0.2623	No
2	10	12	113	76.1	0.1590	No
4	10	12	132	83.2	0.0927	No
7	10	12	186	154	0.6682	No
9	10	12	212	222	0.7667	No
11	10	11	224	243	0.5495	No
14	9	11	225	286	0.2240	No
16	9	7	221	252	0.2991	No
18	9	5	164	275	0.2977	No
21	7	3	225	156	0.9999	No
23	7	3	253	232	0.9999	No
25	6	3	168	243	0.7143	No

Table 3.7 Data and statistical analysis results for tumour volumes of mice treated with 4 fractions of 4 Gy of XR at 9 p.m. vs. the 9 p.m. control group.

Day	Number of mice		Medians (mm ³)		P value	P < 0.05?
	XR	Control	XR	Control		
0	11	13	72.8	61.4	0.8620	No
2	11	13	90.1	72.8	0.8393	No
4	11	13	125	109	0.3848	No
7	11	13	142	155	0.3538	No
9	11	13	125	164	0.0822	No
11	10	12	156	190	0.1380	No
14	10	11	155	283	0.0528	No
16	10	11	154	483	0.0291	Yes
18	10	10	224	417	0.0052	Yes
21	10	8	193	683	0.0031	Yes
23	9	7	208	712	0.0907	No
25	8	5	232	314	0.2844	No

Table 3.8 Data and statistical analysis results for tumour volumes of mice treated with 4 fractions of 4 Gy of XR at 3 a.m. vs. the 3 a.m. control group.

Day	Number of mice		Medians (mm ³)		P value	P < 0.05?
	XR	Control	XR	Control		
0	9	11	66.6	98.3	0.4942	No
2	9	11	71.0	117	0.0403	Yes
4	9	11	85.1	147	0.1966	No
7	9	11	87.4	218	0.0185	Yes
9	9	11	80.1	325	0.0011	Yes
11	9	9	91.0	304	0.0040	Yes
14	9	5	104	343	0.0120	Yes
16	9	5	105	329	0.0120	Yes
18	9	4	152	506	0.0040	Yes
21	8	4	192	613	0.0040	Yes
23	8	3	186	811	0.0121	Yes
25	8	3	196	874	0.0121	Yes

Figures 3.1 – 3.4 show plots of the median tumour volumes that were analysed by the Mann Whitney test as shown in Tables 3.1 – 3.8. Note that no error bars are shown since the plots are of the median values. Data points that were significantly different from their matched control ($p < 0.05$) are indicated by open symbols.

Figure 3.1 Median tumour volumes of mice treated at 9 a.m. and of mice in the 9 a.m. control group.

Figure 3.2 Median tumour volumes of mice treated at 3 p.m. and of mice in the 3 p.m. control group

Figure 3.3 Median tumour volumes of mice treated at 9 p.m. and of mice in the 9 p.m. control group

Figure 3.4 Median tumour volumes of mice treated at 3 a.m. and of mice in the 3 a.m. control group

3.2 Body Weights

Body weight was the first method used to assess the normal tissue toxicity associated with the treatment. Changes in body weight were used since body weight generally reflected the overall well being of the mice. The body weight results for both the mice treated with TPT and those treated with XR in general were not significantly different from those of their matched controls. Tables 3.9, 3.10, 3.11 and 3.12 show the results of the statistical analysis for the body weights of the mice given Topotecan vs. their matched control. Table 3.11 is the only table that contains a point that was significantly different from its matched control.

Table 3.9 Data and statistical analysis results for body weights of mice treated with 12 mg/kg of TPT at 9 a.m. vs. the 9 a.m. control group.

Day	Number of mice		Medians (g)		P value	P < 0.5?
	TPT	Control	TPT	Control		
0	14	14	24.8	24.3	0.1827	No
4	14	14	24.9	24.1	0.3702	No
7	14	14	24.4	24.7	0.5503	No
11	13	12	24.5	24.5	0.9566	No
14	13	9	24.7	24.4	0.2563	No
18	8	5	25.8	24.3	0.1939	No
21	8	3	26.7	25.2	0.4970	No
25	7	3	27.4	24.9	0.1833	No

Table 3.10 Data and statistical analysis results for body weights of mice treated with 12 mg/kg of TPT at 3 p.m. vs. the 3 p.m. control group.

Day	Number of mice		Medians (g)		P value	P < 0.5?
	TPT	Control	TPT	Control		
0	15	12	26.4	25.6	0.8072	No
4	15	12	25.9	25.3	0.8262	No
7	15	12	25.8	25.5	0.7326	No
11	15	11	25.1	25.5	0.7555	No
14	15	11	24.7	25.5	0.3637	No
18	12	5	24.5	25.3	0.6135	No
21	11	3	26.0	26.6	0.9999	No
25	5	3	25.9	26.5	0.9999	No

Table 3.11 Data and statistical analysis results for body weights of mice treated with 12 mg/kg of TPT at 9 p.m. vs. the 9 p.m. control group.

Day	Number of mice		Medians (g)		P value	P < 0.5?
	TPT	Control	TPT	Control		
0	13	13	24.8	22.5	0.0858	No
4	13	13	24	23.3	0.3427	No
7	13	13	23.8	23.1	0.6444	No
11	13	12	22.8	24.4	0.1824	No
14	13	11	21.9	23.8	0.0489	Yes
18	11	10	22.4	24.6	0.2750	No
21	11	8	23.5	23.7	0.6795	No
25	9	5	24.2	23.8	0.7972	No

Table 3.12 Data and statistical analysis results for body weights of mice treated with 12 mg/kg of TPT at 3 a.m. vs. the 3 a.m. control group.

Day	Number of mice		Medians (g)		P value	P < 0.5?
	TPT	Control	TPT	Control		
0	13	11	24.6	23.6	0.1396	No
4	13	11	23.3	23.1	0.4513	No
7	13	11	23.1	23.1	0.5429	No
11	12	9	22.2	24.1	0.1099	No
14	11	5	21.5	23.1	0.0700	No
18	11	4	22.6	24.3	0.1508	No
21	11	4	23.5	23.6	0.6952	No
25	9	3	23.6	25.1	0.4818	No

Tables 3.13 – 3.16 contain the data and statistical analysis for the body weights of those mice that were treated with XR. The only time point at which there was any significant difference was when the mice were treated with X-radiation at 9 a.m. However, on all three of the days when there was a significant difference the treated group had the greater median body weight. Therefore, the body weights for the mice treated with X-radiation do not indicate any serious adverse effects on the normal tissue.

Table 3.13 Data and statistical analysis results for body weights of mice treated with 4 fractions of 4 Gy of XR at 9 a.m. vs. the 9 a.m. control group.

Day	Number of Mice		Medians (g)		P value	P < 0.05?
	XR	Control	XR	Control		
0	8	14	26.9	24.3	0.0009	Yes
4	8	14	25.5	24.1	0.0704	No
7	8	14	26.7	24.7	0.0222	Yes
11	8	12	25.8	24.5	0.1533	No
14	7	9	26.3	24.4	0.0033	Yes
18	7	5	26.6	24.3	0.0667	No
21	6	3	26.5	25.2	0.2619	No
25	6	3	26.6	24.9	0.2619	No

Table 3.14 Data and statistical analysis results for body weights of mice treated with XR at 3 p.m. vs. the 3 p.m. control group.

Day	Number of Mice		Medians (g)		P value	P < 0.05?
	XR	Control	XR	Control		
0	10	12	26.5	25.5	0.0993	No
4	10	12	25.8	25.3	0.4096	No
7	10	12	26.8	25.4	0.1134	No
11	10	11	25.9	25.5	0.5973	No
14	9	11	26.6	25.5	0.2389	No
18	9	5	26.5	25.3	0.2091	No
21	7	3	26.8	26.6	0.5167	No
25	6	3	27.4	26.5	0.7143	No

Table 3.15 Data and statistical analysis results for body weights of mice treated with 4 fractions of 4 Gy of XR at 9 p.m. vs. the 9 p.m. control group.

Day	Number of Mice		Medians (g)		P value	P < 0.05?
	XR	Control	XR	Control		
0	11	13	24.1	22.5	0.5239	No
4	11	13	23.2	23.3	0.9077	No
7	11	13	23.2	23.1	0.8167	No
11	10	12	24.2	24.4	0.7666	No
14	10	11	23.2	23.8	0.6471	No
18	10	10	23.3	24.6	0.6842	No
21	10	8	23.5	23.6	0.9654	No
25	8	5	23.3	23.8	0.7242	No

Table 3.16 Data and statistical analysis results for body weights of mice treated with 4 fractions of 4 Gy of XR at 3 a.m. vs. the 3 a.m. control group.

Day	Number of Mice		Medians (g)		P value	P < 0.05?
	XR	Control	XR	Control		
0	9	11	24.3	23.6	0.1192	No
4	9	11	24.5	23.1	0.0874	No
7	9	11	24.3	23.1	0.224	No
11	9	9	24.6	24.1	0.4363	No
14	9	5	24.6	23.1	0.4376	No
18	8	4	24.7	24.3	0.4606	No
21	8	4	25.0	23.6	0.3677	No
25	8	3	24.5	25.1	0.6303	No

Figures 3.5 – 3.8 contain all of the median body weight values. Significantly different values are indicated with an open symbol. The plots are of median body weights; therefore, there are no error bars plotted.

Figure 3.5 Median body weights of mice treated at 9 a.m. and of mice in the 9 a.m. control group.

Figure 3.6 Median body weights of mice treated at 3 p.m. and of mice in the 3 p.m. control group.

Figure 3.7 Median body weights of mice treated at 9 p.m. and of mice in the 9 p.m. control group.

Figure 3.8 Median body weights of mice treated at 3 a.m. and of mice in the 3 a.m. control group.

3.3 Leukocyte Counts

The number of leukocytes present in peripheral whole blood was the second parameter used to assess the normal tissue toxicity.

Tables 3.17-3.20 contain the data for the mice that were treated with TPT. Table 3.17 shows that there was a statistically significant difference between treated mice and control mice on all four of the days when blood was taken for those mice in the 9 a.m. group. The median number of leukocytes was lower on all 4 days in the treated group. Table 3.18 indicates that there was a statistically significant difference in the number of leukocytes present in the blood of those mice in the 3 p.m. group on days 3, 10 and 17 (i.e. weeks 1, 2 and 3 of treatment). In each of these cases, the median number of leukocytes was less in the treated group compared to the control group. Table 3.19 which contains the data for those mice treated with TPT at 9 p.m. shows that there was a significant difference between the two groups only once out of the 4 times when blood was taken, and that occurs on day 10. Table 3.20 shows that there was no significant difference in leukocyte counts between those mice treated with TPT at 3 a.m. compared with the 3 a.m. control group.

Table 3.17 Data and statistical analysis for leukocyte counts of mice treated with 12 mg/kg of TPT at 9 a.m. vs. the 9 a.m. control group.

Day	Number of mice		Medians (leukocytes/mm ³)		P value	P < 0.05?
	TPT	Control	TPT	Control		
3	14	14	7825	11275	0.0159	Yes
10	12	12	7325	11300	0.0194	Yes
17	8	9	9075	17350	0.0006	Yes
24	7	3	7050	19600	0.0333	Yes

Table 3.18 Data and statistical analysis for leukocyte counts of mice treated with 12 mg/kg of TPT at 3 p.m. vs. the 3 p.m. control group.

Day	Number of mice		Medians (leukocytes/mm ³)		P value	P < 0.05?
	TPT	Control	TPT	Control		
3	15	12	8800	13450	0.0059	Yes
10	15	11	6700	10350	0.0224	Yes
17	12	10	8250	11600	0.0041	Yes
24	5	3	7700	6000	0.9999	No

Table 3.19 Data and statistical analysis for leukocyte counts of mice treated with 12 mg/kg of TPT at 9 p.m. vs. the 9 p.m. control group.

Day	Number of mice		Medians (leukocytes/mm ³)		P value	P < 0.05?
	TPT	Control	TPT	Control		
3	13	13	2750	5800	0.137	No
10	13	12	4200	8025	0.018	Yes
17	11	11	5550	2250	0.066	No
24	5	5	5250	7250	0.5476	No

Table 3.20 Data and statistical analysis for leukocyte counts of mice treated with 12 mg/kg of TPT at 3 a.m. vs. the 3 a.m. control group.

Day	Number of mice		Medians (leukocytes/mm ³)		P value	P < 0.05?
	TPT	Control	TPT	Control		
3	13	11	4450	4950	0.3062	No
10	13	9	3750	3050	0.4048	No
17	11	5	4050	2150	0.1333	No
24	7	3	3800	7100	0.5273	No

Tables 3.21 – 3.24 contain the data for leukocyte counts of the mice treated with X-Radiation. Table 3.21 contains the data for mice treated at 9 a.m. On the first three of the four days when blood was collected there was a significant reduction in the number of leukocytes present in the treated group. Table 3.22 contains the data for mice treated at 3 p.m. On three of the four days tested the number of leukocytes present was significantly reduced among the mice in the treated group. Table 3.23 contains the data for mice treated at 9 p.m. For those mice treated with XR at 9 p.m. there was a significant effect on the number of leukocytes present on the first two of the four days tested. Table 3.24 contains the data for leukocyte counts of those mice that were treated with XR at 3 a.m. There was only one day when there was a significant difference between the treated group and the untreated control, day 10, however, the treated group actually had a greater number of leukocytes present in the blood so the treatment did not have an adverse effect on the mouse at that time.

Table 3.21 Data and statistical analysis for leukocyte counts of mice treated with 4 fractions of 4 Gy of XR at 9 a.m. vs. the 9 a.m. control group.

Day	Number of mice		Medians (leukocytes/mm ³)		P value	P < 0.05?
	XR	Control	XR	Control		
3	8	14	8125	11275	0.0374	Yes
10	8	12	7025	11300	0.0151	Yes
17	7	9	7750	17350	0.0002	Yes
24	6	3	12175	19600	0.1667	No

Table 3.22 Data and statistical analysis for leukocyte counts of mice treated with 4 fractions of 4 Gy of XR at 3 p.m. vs. the 3 p.m. control group.

Day	Number of mice		Medians (leukocytes/mm ³)		P value	P < 0.05?
	XR	Control	XR	Control		
3	10	12	7275	13450	0.0010	Yes
10	10	11	7400	10350	0.0083	Yes
17	9	10	6100	11600	0.0010	Yes
24	6	3	8475	6000	0.5476	No

Table 3.23 Data and statistical analysis for leukocyte counts of mice treated with 4 fractions of 4 Gy of XR at 9 p.m. vs. the 9 p.m. control group.

Day	Number of mice		Medians (leukocytes/mm ³)		P value	P < 0.05?
	XR	Control	XR	Control		
3	11	13	4100	5800	0.0345	Yes
10	10	12	4175	8025	0.0069	Yes
17	10	11	3850	2250	0.2908	No
24	8	5	5175	7250	0.0932	No

Table 3.24 Data and statistical analysis for leukocyte counts of mice treated with 4 fractions of 4 Gy of XR at 3 a.m. vs. the 3 a.m. control group.

Day	Number of mice		Medians (leukocytes/mm ³)		P value	P < 0.05?
	XR	Control	XR	Control		
3	9	11	4800	4950	0.8792	No
10	9	9	6150	3050	0.0142	Yes
17	9	5	4300	2150	0.0829	No
24	8	3	5500	7100	0.4970	No

Figures 3.9 – 3.12 contain all of the values for median leukocyte counts. Open symbols represent points that are significantly different from their matched control ($p < 0.05$). Again, no error bars are plotted since the values are for the median number of leukocytes.

Figure 3.9 Median leukocyte counts of mice treated at 9 a.m. and of mice in the 9 a.m. control group

Figure 3.10 Median leukocyte counts of mice treated at 3 p.m. and of mice in the 3 p.m. control group.

Figure 3.11 Median leukocyte counts of mice treated at 9 p.m. and the 9 p.m. control group.

Figure 3.12 Median leukocyte counts of mice treated at 3 a.m. and the 3 a.m. control group.

Chapter 4

Discussion

4.1 Tumour Volumes

For mice treated with TPT at 9 a.m. there was only point that was significantly different, day 18 (Table 3.1). This might have been simply a random occurrence and as such, the treatment was not deemed as being effective at this time. For mice given the same treatment at 3 p.m. the only day that showed a significant difference was day 4, however, it was actually the treated group that had the larger median tumour volume. Since this occurred early in the experiment it was likely that this was a result of the treated group having slightly larger tumours at the beginning of the experiment. Therefore, the TPT treatment was not considered effective when administered at 3 p.m. For mice treated at 9 p.m. there were 3 days, days 16, 18 and 21 (Table 3.3), when the median tumour volumes of the treated mice were smaller than the matched control. When delivered at 9 p.m. the TPT treatment was effective at reducing tumour size. For the mice treated with TPT at 3 a.m. there were seven days when the median tumour volumes of the treated group were smaller than the matched control group, days 9, 14, 16, 18, 21, 23 and 25 (Table 3.4). The TPT treatment was therefore the most effective at reducing tumour volume when delivered at 3 a.m. (21 HALO) among the four times that were tested. This was during the late active phase of the mice and is contrary to the

initial hypothesis that TPT would be most effective when delivered during the rest phase of the mice.

Although these results were not as predicted, they are reasonable based on other previous findings. For rats, it has been shown that tumour tissue blood flow increased significantly during the night time (Hori, 1996). Dexorubicin hydrochloride (ADM) had a greater therapeutic effect on rats when administered at 16 HALO (early active phase) then when administered at 4 HALO (early rest phase) (Hori, 1996). This increase in therapeutic gain may be a result of an increase in the delivery of the drug to the tumour tissue as a result of the circadian increase in tumour tissue blood flow (Hori, 1996). It has also been shown for two different human colorectal tumour xenographs in nude mice that the time of greatest tumour blood flow was at 17 HALO (Blumenthal et al., 2000). The results of this experiment suggest that tumour tissue blood flow may have had an impact on the effectiveness of Topotecan, since the time of greatest treatment efficacy was at 3 a.m. (21 HALO). Tumour tissue blood flow may be the main contributor to the chronomodulated effect of TPT and not the circadian variation in S-Phase fraction of tumour cells as was originally hypothesized.

For mice treated with x-radiation at 9 a.m., there were 3 days when the treated group had significantly smaller tumour volumes than its matched control, days 9, 16, 18. On all three of these days the treated group had the smaller median tumour volume. This result indicated that the treatment was somewhat effective at reducing tumour volume when delivered at 9 a.m.

For those mice treated with XR at 3 p.m. there was no significant difference between the treated group and its matched control on any of the days treated, therefore, the treatment was not effective when delivered at 3 p.m.

Mice treated with XR at 9 p.m. showed a similar trend as those mice treated at 9 a.m. For mice treated at 9 p.m. there were three significant points, days 16, 18 and 21 (Table 3.6), therefore at 9 p.m. the treatment was somewhat effective. For those mice treated with x-radiation at 3 a.m. the tumour volumes of the treated group were significantly different than their matched control on 10 of the 12 days when tumour volumes were measured. This indicated that the treatment with x-radiation was the most effective when delivered at 3 a.m. This was during the active phase of the mice as it was with the TPT treatment. However, in this instance the finding agrees with the initial hypothesis that x-radiation would be the most effective when delivered during the active phase. It was hypothesized that the greatest number of tumour cells would be in mitosis during this time and therefore cause the tumour to be more radiosensitive. In addition, it is known that oxygen increases radiosensitivity (Haus, 2002). If tumour tissue blood flow was the greatest during the active phase, this increase in oxygen present in the tumour may also have contributed to the chronomodulated effect.

It is interesting to consider only the control tumour volumes for all of the time points. From Figures 3.1 – 3.4 it appears as though the control mice in both the 9 a.m. and 3 p.m. groups had somewhat smaller tumour volumes than the mice in the 9 p.m. and 3 a.m. groups. This difference was likely due to the fact that the mice were injected with tumour cells at different circadian times in the regular room and light-shifted room. All mice were injected at 10:00 a.m.; however, in the light-shifted room 10:00 a.m. was

effectively 10:00 p.m. There is data that suggests that the circadian time of tumour cell injection effects the rate of tumour take as well as the eventual tumour size (Hrushesky, et al., 1999). Although there was a difference among the control groups this should not have affected the experiment because the mice that were treated were injected with tumour cells at the same time as their matched control. This is an important piece of evidence that supports the need for a time-matched control for this type of experiment.

4.2 Body Weights

For both topotecan (SmithKline Beecham Inc., 1997) and x-radiation bone marrow is the dose limiting tissue. Consequently, it is not surprising that there was very little change in body weight for any of the mice throughout the experiment. A significant reduction in body weight for several days or time points of the experiment would have been an indication that the dose being administered was too large. For those mice treated with TPT there was only one point among all four time points when there was a significant difference between a treated group and its matched control which occurred on day 14 in the group treated with TPT at 9 p.m. Since this was only a one-time occurrence, it was not considered an indicator of a toxic effect at that time. For those mice treated with x-radiation, there was only one group that demonstrated a significant difference between a treated group and its matched control: the group treated at 9 a.m. For the mice treated at that time there were three days when the medians body weights were significantly different, days 0, 7 and 14, however, on all 3 of these days it was the

treated group that has the larger median body weight. Therefore, this was only a consequence of the treated group being larger at the beginning of the experiment. This occurred despite the use of age-matched controls and is a good example of the natural variation that occurs in an experiment when dealing with animals. This is not a sign that the treatment was toxic and it was therefore concluded that the body weights do not indicate any significant toxicity.

An interesting point to note is that the body weights in the 9 p.m. and 3 a.m. groups were somewhat lower than those in the 9 a.m. and 3 p.m. groups. The groups were all weighed at 10 a.m. regular time. For the light-shifted group 10 a.m. corresponded to 10 p.m. or 16 HALO, which was the early active phase of the mice, which means that they had just begun to eat their food for the day. Since 10 a.m. or 4 HALO was the early rest phase when the mice had finished eating for the day and were beginning to sleep, it was not surprising that the mice were slightly heavier after having eaten than prior to eating.

4.3 Leukocyte Counts

The overall trend for leukocyte counts was the same for those mice treated with TPT and those that were irradiated. For both treatment with TPT and with XR there were a total of 8 days when the number of leukocytes present in the blood was significantly reduced.

For those mice treated with TPT at 9 a.m. on all four days during the experiment when blood was collected the treated group had significantly fewer leukocytes present. For mice treated with TPT at 3 p.m. on three of the four days with blood samples were taken there were significantly fewer leukocytes present in the blood of the treated mice. For mice treated with TPT at 9 p.m. there was only one day when the number of leukocytes present was significantly reduced in the treated group, therefore, the treatment had only a slight adverse effect on the mice when delivered at 9 p.m. When the TPT treatment was delivered at 3 a.m. there was no significant reduction in the number of leukocytes present on either of the four days tested.

Based on these results the TPT treatment has no significant effect on the number of leukocytes present in the blood at 3 a.m. This is contrary to the hypothesis that the TPT treatment would be the most toxic during the active phase. However, since the tumour volumes showed that 3 a.m. was the most effective time to deliver TPT it was not surprising that it was also the time of least normal tissue toxicity. It was anticipated that the time of greatest treatment efficacy would also be the time of least normal tissue toxicity based on previous findings with other drugs (Levi, 1997).

For mice treated with x-radiation at 9 a.m. on three of the 4 days tested there were significantly fewer leukocytes present in the blood of the mice in the treated group. This indicated that the irradiation was harmful to the mice when delivered at 9 a.m. For mice irradiated at 3 p.m. the trend was the same; on three of the four days the number of leukocytes in the treated group was significantly reduced. Therefore, the x-radiation treatment was also harmful when delivered at 3 p.m. When delivered at 9 p.m. the x-radiation treatment was slightly less harmful since there were only two days when the

number of leukocytes was reduced. The group of mice treated with x-radiation at 3 a.m. showed no effect on the number of blood leukocytes present because of the treatment. There was one point in this group when there was a significant difference between the treated group and its matched control; however, it was the control group that had the lower leukocyte count. This was the result of a random discrepancy between the two groups. Therefore, it was concluded that the least harmful time to administer the XR treatment was at 3 a.m. This agrees with the initial hypothesis that x-radiation would be the least harmful when delivered during the rest phase of the mice. It is also worth noting that this was also the time of greatest treatment efficacy for mice treated with XR. It was predicted that the time of greatest treatment efficacy would also be the time of least normal tissue toxicity, which was the case here.

Leukocyte counts were lower at 9 p.m. and 3 a.m. among both treated and control mice (Figures 3.11 and 3.12) than at 9 a.m. and 3 p.m. (Figures 3.9 and 3.10). The mice in the 9 p.m. and 3 a.m. groups were the mice that were maintained on a reverse light cycle. The lower leukocyte counts among the mice in the light-shifted group could be due to a variety of factors. Those blood samples were taken under red light so as not to disrupt the light-dark pattern of the mice and were therefore more difficult to collect. Perhaps the amount of blood collected in the dark was lower than that collected in the light. It is also possible that there was a circadian variation in the number of leukocytes present in the blood. This circadian variation may have been due to the hormone melatonin. Melatonin levels in the body vary throughout the day and melatonin plays a role in immune function (Reiter, 2003). Whatever the reason, this variation in the control leukocyte counts again illustrates the need for a matched control for each time point.

Without a matched control we would have compared all of the measurements to one control group, most likely one that would have been maintained on a regular light cycle. This would have been the wrong decision because the blood counts would have appeared much too low in the dark room.

Chapter 5

Conclusion

The time of treatment delivery does contribute to the effectiveness of treatment for both topotecan and x-radiation when given to nude mice bearing human colorectal tumour xenographs. For both topotecan and x-radiation, it was during late active phase, 3 a.m. (21 HALO), which was the best time to deliver the treatment. The amount of normal tissue toxicity was the lowest and the amount of tumour kill was the greatest. The rest phase, 9 a.m. (3 HALO) was the worst time to deliver the treatment in both cases. The amount of normal tissue toxicity was the highest and the amount of tumour kill was the lowest.

The time of least normal tissue toxicity was also the time of best treatment efficacy. This agrees with other findings (Levi, 1997; Peters et al., 1987; Sothorn et al., 1989). This has great implications when considering patient treatment planning. It may be possible to significantly escalate the dose administered without significantly increasing the harmful normal tissue effects by delivering treatment during specific times of the corresponding active phase of humans.

In summary, hypothesis 1 was incorrect and hypothesis 2 was correct. TPT given during the active phase (not the rest phase as predicted) resulted in decreased normal tissue toxicity and increased tumour efficacy. XR given during the active phase resulted in decreased normal tissue toxicity and increased tumour efficacy.

5.1 Future Research

1. The next step for this project is to combine the two different types of treatment, i.e. to treat with both TPT and x-radiation at a given time. There are virtually no data in the literature on the potential benefits of chronomodulated chemoradiation treatments. It should be noted that chemoradiation treatments are very important in the treatment of cancer. The hypothesis here is that giving the combined treatment at a particular time during the active phase of the mice would lead to maximum efficiency of both TPT and XR.
2. Measure the tumour blood flow to determine whether there is an increase in blood flow as expected at 3 a.m. This can be done using MRI techniques.
3. Determine the melatonin levels in the blood serum of the mice at various times to determine if the time of greatest melatonin concentration corresponds to the time when the greatest number of leukocytes are present in the blood.

This work can also potentially lead to manipulating the circadian rhythm of patients so that treatments are given at the optimum time according to chronotherapy considerations.

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