

CHARACTERIZATION OF MATLYLU PROSTATE CANCER CELLS TO
COMBINED TREATMENT WITH APOCYNIN AND RADIATION

THE IN VITRO AND IN VIVO CHARACTERIZATION OF MATLYLU PROSTATE
CANCER CELL LINE TO COMBINED TREATMENT WITH THE NADPH
OXIDASE INHIBITOR APOCYNIN AND IONIZING RADIATION

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Abstract

It has been observed that the radiation response of a cell line may positively correlate with baseline levels of reactive oxygen species (ROS) generation, which is facilitated, in part, by the function of NADPH oxidase membrane proteins. Apocynin has been successfully used to inhibit ROS generation via NADPH oxidase for other uses. Here, the effectiveness of apocynin to improve sensitivity to radiation in the MATLyLu cell line was examined. MATLyLu-implanted Cop/Hsd rats undergoing 10Gy/5fx radiation treatment demonstrated improved tumour control, as measured by survival time (to predetermined endpoints), when given apocynin orally throughout their radiation treatment, but not when given apocynin only before radiation. Rats given apocynin without radiation did not demonstrate improved survival over controls. MATLyLu cells given apocynin *in vitro*, however, did not demonstrate improved response to radiation or a reduction in baseline ROS production on a per cell basis, and apocynin was found to inhibit growth when given alone. These contradictory *in vivo* and *in vitro* findings may be explained by certain proposed mechanisms of action for apocynin which requires it to be first synthesized into its dimer, diapocynin, before it will inhibit NADPH oxidase, and this synthesis may occur during ingestion. Further investigations with diapocynin and the MATLyLu cell line are required. Therefore the mechanism is uncertain, but it was concluded that apocynin and radiation administered together *in vivo* improves radiation response as compared to either treatment alone.

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Introduction

The effect of apocynin on cell and tissue radiosensitivity involves numerous concepts. Low-LET (linear energy transfer) radiation damages cells primarily by the production of reactive oxygen species, which is an effect that can be mitigated by sensitizing compounds and techniques, or can also be resisted by cells and tissues which have been pre-exposed to a similar stressor by a mechanism known as the adaptive response. Pre-exposed cells and tissues often have upregulated defence mechanisms to the particular stressor, and thus cells with no previous exposure would be relatively deficient in their defences. NADPH oxidase is responsible for a share of the reactive oxygen species produced in cells and tissues. Inhibiting NADPH oxidase results in a decrease in reactive oxygen species production, and thus a decrease in the appropriate defence mechanisms. Apocynin is a potent NADPH oxidase inhibitor with the potential to increase radiosensitivity in cells and tissues by the process outlined above. A discussion of each concept in greater detail is to follow.

The Physics of Low-LET Radiotherapy

Radiation destroys cancer cells as well as healthy cells primarily by damaging their DNA ¹. In rapidly dividing tissue, such as tumour cells, cell “death” is said to occur when the cell become incapable of further division. Thus whether DNA damage causes proliferative cells to autolyse by apoptosis, or by simply rendering the cell incapable of successfully cycling through mitosis is inconsequential in tumours – tumour “death” occurs in either situation ². However, not all cells that sustain DNA damage are destined to die. DNA repair processes allow cells to often survive radiation-induced DNA damage unharmed. Single-strand breaks (SSBs) are typically the most prevalent form of DNA strand breaks, and are easily repaired.¹. When two SSBs are near each other, or following a larger ionization event, double-strand breaks (DSBs) may form, where the DNA is completely severed. DSBs can be repaired by homologous recombination repair (HRR) or non-homologous end-joining (NHEJ). NHEJ is a four-step error-prone process that attempts to reattach two ends of broken DNA. If multiple DSBs occur in reasonably close proximity, there is the potential for NHEJ to join mismatched segments of DNA, forming chromosomal aberrations such as translocations, rings, anaphase bridges, dicentric and

acentric fragments (dicentrics), and small deletions. Rings, anaphase bridges, and dicentrics are lethal to the cell, as they will prevent successful division. However translocations and small deletions are not necessarily lethal, and may lead to mutation if the chromatid changes occurred in a gene coding DNA sequence ².

These aberrations are caused by radiation by one of two forms of interaction; the direct effect, or the indirect effect, which differ in probability based on the quality and type of radiation. The direct effect is the term used to describe the event of DNA interacting directly with the incident radiation, resulting in a DNA break. This form of DNA damage is predominantly the effect of charged particles which deposit their energy intensely along a short track, described as high-LET (linear energy transfer) radiation ¹. LET describes the amount of energy deposited by a type of radiation along a unit of distance. The indirect effect is more attributable to low-LET radiation such as gamma- and x-rays. These rays are more sparsely ionizing produce fast, secondary particles (mostly electrons), which interact with the cellular medium, mostly composed of water. The interaction of fast electrons with water results in the radiolysis of water molecules, producing water ions, H_2O^- and H_2O^+ which rapidly breakdown into more ions such as OH^- or H^+ , as well as molecules with unpaired valence electrons, known as free radicals, such as $\text{OH}^\cdot$ and $\text{H}^\cdot$ ³. These ions and free radicals may neutralize each other, forming stable water, or they may form toxic hydrogen peroxide, or they may interact with DNA base-pairs and cause DNA damage ⁴. The indirect effect, therefore, involves a chain of events: The incident photon produces a fast electron, which then forms water ions, which

then form hydroxyl and hydrogen ions and free radicals, which may migrate to the DNA and cause strand breaks. Because of the multiple steps involved in this process, it is therefore possible to mitigate or enhance these effects at multiple stages. Approximately two-thirds of DNA damaged produced by x-rays and gamma-rays are produced by this method ².

In external-beam radiation therapy of internal tumours, gamma- and x-irradiation are most commonly used, as their low-LET allows for greater penetrability, and use of multiple beams can generate very precise high-dose areas in the body. Because the indirect effect predominates with these types of radiation, and because the indirect effect is subject to chemical sensitization and protection, its thorough understanding is crucial to the development of new radiotherapeutic sensitization and protective techniques.

Existing methods of affecting indirect effect radiosensitization include oxygen administration (or deprivation), chemical radiosensitizers and chemical radioprotectants⁴, as well as hyperthermia¹. The oxygen enhancement effect is the oldest, simplest, and perhaps most effective means of radiosensitization. It was discovered in 1912 by Swartz, who noted an inhibited inflammatory response from radium when the applicator was pressed firmly against his skin². This effect, quantified by what is known as the ‘oxygen enhancement ratio’, has been shown to require that for sensitization, oxygen be available not necessarily during but immediately after irradiation (within 5ms of irradiation). The hypothesis that describes the need for prompt oxygen availability also explains why this effect is not observed in densely-ionizing high-LET irradiations: DNA damage produced

by free radicals may be quickly repaired in a hypoxic environment, however an oxygen molecule can bind to a site of free-radical-induced DNA damage, “fixing” it, or making it permanent².

A family of radiation sensitizers called nitroimidazoles preferentially increases the potency of the indirect radiation effects⁴. Nitroimidazoles are oxygen-mimicking agents that may diffuse further into tissue than oxygen from an available blood supply, therefore reaching deeper into hypoxic tissues. However these compounds have not been clinically implemented due to negative side effects, and because they are not exclusively taken up in malignant tissue⁴.

A different approach to increasing tumour control in radiation therapy has been to provide chemical protection to regular tissue, rather than sensitization to malignant tissue, and then increase the given dose to the tumour while sparing normal tissue. The most widely used family of radioprotectants are sulfhydryls, which act as free radical scavengers that compete with oxygen for free radicals formed during the radiolysis of water⁴, or by donating a hydrogen atom to mediate chemical repair at the site of DNA damage².

Another technique that is critical to successful radiotherapeutic tumour control is fractionation. Fractionation is the practice of delivering a given dose of radiation in smaller doses given at intervals (typically daily, in the clinical environment), rather than all at once. A fractionated dose regiment is less efficient than a single dose at controlling tumours, in that two 500cGy doses separated by time cause less damage than a single

dose of 1000cGy. This is less practical from a patient throughput perspective; however, fractionating the dose achieves many crucial goals, characterized by “the four R’s”, repair, reassortment, reoxygenation, and repopulation⁴.

Repair immediately follows irradiation by the mechanisms outlined earlier, and is responsible for the “shoulder” region that is characteristic of log-graphed radiation survival curves. Tumours are typically less capable of undergoing repair than surrounding tissues because oxygen is required to conduct repair processes and tumours often outgrow their blood supply, as is explained in more detail for the reoxygenation mechanism. Reassortment (or redistribution) describes the continued mitotic cycling of cells that survived irradiation. This is important because during irradiation, some cells in an asynchronous population will be in more radiosensitive stages of mitosis than others (G2, M). Reassortment allows for cells that were previously in a resistant stage of mitosis to cycle into a more sensitive stage and be more susceptible to elimination during the next fraction. Reoxygenation describes how tissues that previously had poor access to oxygen (and were therefore relatively radioresistant) may gain access in between fractions. This is important because many tumours quickly outgrow their blood supply, and the cells nearer the centre are starved of oxygen and have higher chances of surviving irradiation. In between fractions, the outer, well-oxygenated (and therefore sensitive) parts of the tumour are killed and sloughed off, allowing the previously hypoxic tissue beneath it renewed access to oxygen before the next fraction. Lastly, repopulation describes the replacement of eliminated tissue by surviving, dividing cells. This is important because radiation can be very hard on healthy tissue as well as tumour tissue. Without

fractionation, radiation therapy would be a significantly less feasible treatment modality because it would cause too much damage to healthy tissue, and it would not be capable of killing tumour tissue that was either hypoxic or in less sensitive stages of mitosis during treatment^{1,2,4}.

Lastly, there are inherent traits of tumour cells that conveniently render them more radiosensitive than healthy tissue, as well as make some of the aforementioned adjuvant treatments possible. In 1906, Bergonié and Tribondeau determined the radiosensitivity of rodent germ cells are varying stages of spermatogenesis. Their findings showed that cells with higher mitotic rate, a long mitotic future, and are poorly differentiated are the most sensitive to radiation. With some exceptions, such as lymphocytes, these laws hold true for most tissues³. Tumour cells are typically very mitotically active, have a mitotic lifespan limited only by the host organism, and are less differentiated than the cells they mutate from. Additionally, their higher mitotic rate allows them to uptake radiosensitizing agents more quickly than normal tissues, and can therefore be targeted for treatment with precise timing⁴. This is also the premise by which PET (positron emission tomography) works, as gamma-emitting radionuclides are attached to sugar molecules which are preferentially metabolised by tumour cells, allowing for their detection³.

In summary, the anti-tumour effect of low-LET ionizing radiation relies on a number of characteristics that present opportunity for unique adjuvant treatments such as oxygen enhancement, chemical radiosensitizers and protectors, which are taken further advantage of by employing a fractionated treatment regimen. The methods outlined above

do not exhaust the ways in which the indirect effect of ionizing radiation can be fully exploited. Others such as androgen-deprivation therapy and NADPH oxidase inhibition will be discussed in the chapters to follow.

Adaptive Response

Adaptive response is the term used to describe the tendency for cells, tissues, or organisms to become resistant to damage from a particular stressor following a prior sublethal exposure to it. Specific examples include induced resistance to arsenic in Zebrafish⁵ and the infamous insecticide p,p'-1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, better known as DDT, in rats⁶, including many oxidative stressors from androgens⁷ chemotherapeutic agents⁸, ionizing radiation^{9,10,11,12,13,14,15} and more. An applicable common analogue for this phenomenon is exercise; organisms exposed to physical stresses such as resistance or exertion become more capable of enduring said challenge in future events, measurable not only by improved performance, but also by decreased indicators of stress such as lipid peroxidation¹⁶.

With regards to oxidative stress, protection is hypothesized to be conferred by the upregulation of antioxidative enzymes that interfere with the production of free radicals that can cause damage to the organism. Manganese superoxide dismutase (MnSOD) catalyzes the dismutation of the superoxide anion into hydrogen peroxide, which is readily neutralized into water and oxygen by catalase. Both catalase and MnSOD are upregulated in tissues in response to oxidative stressors such as ozone¹⁷, hydrogen

peroxide¹⁸, butylated hydroxytoluene (BHT)¹⁹, chromium²⁰, and even conscious physical restraint²¹. Clusterin, a protein associated with apoptosis inhibition, is also upregulated by the very oxidative stressors it protects against²².

Because these stressors induce DNA damage in similar fashions and induce upregulation of the same antioxidizing mechanisms in the adaptive response, the induced resistance is common to all oxidative stressors. Indeed, a wide range of oxidative stressors have been demonstrated to induce cross-resistance, including gamma and x-irradiation, heat shock, aldehyde and heavy metal exposure, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-ethylmaleimide, heme¹¹, chemotherapy agents mitomycin C, bleomycin, chemosterilant quinaquine dihydrochloride⁸, cisplatin, as well as UV radiation¹³.

In studies that have demonstrated improved survival to a large ‘challenge’ dose after being initially exposed to a smaller ‘priming’ dose, it has been shown that this effect is due to an induced resistance and not an increase in proliferation⁸, although it has also been shown that the priming dose can trigger a differentiation process that does allow for greater proliferation before and/or after the challenge dose in patients receiving whole-body radiotherapy²³. Hypothetically, differentiation to a less proliferative cell should be possible as well.

In addition to survival and proliferation, adaptive response has been measured in terms of organ function *in vivo*¹⁵, acute radiation syndrome (ARS) resulting in death²³ chromosomal aberrations, mutation induction, radiosensitivity, and DNA repair¹⁴.

Adaptive response to radiation has been demonstrated by the difference between chromosome aberration sensitivity in nuclear power workers (chronic exposure) and Japanese atomic bomb survivors (acute exposure), where those chronically exposed were up to six times more resistant¹⁰. Similarly, blood samples from residents of the high-background radiation area (HBRA) Ramsar, Iran showed 56% fewer chromosomal abnormalities following a challenge dose of radiation, when compared to blood samples of inhabitants of neighbouring low-level areas⁹. The background dose in this HBRA reaches nearly 300mSv/year – approximately one hundred times the average background exposure in North America.

It has also been shown that constantly elevated levels of reactive oxygen species (ROS) may play a crucial role in maintaining oxidative resistance¹⁴, which is consistent with the idea of radioresistance in individuals living in HBRA.

It is beyond the scope of this thesis, but interesting to consider the general importance of having elevated levels of defence against oxidative stress. While it is known that large stressors, taking radiation for example, are harmful, there is an enormous and growing body of evidence in support of the theory of ‘Hormesis’, whereby small exposures to known mutagens, toxins, and teratogens give way to beneficial effects. The most interesting case of this occurred in Taiwan in the early 1980s when a series of

buildings were erected using beams made from steel that had been contaminated with radioactive cobalt-60. This resulted in 10,000 occupants receiving an average of 72mGy/year²⁴ (over 20 times the North American natural background²). During those years, the affected individuals showed an overall cancer mortality rate equal to 3% of the general Taiwanese public. Similarly, inhabitants of HBRAs in Ramsar, Iran and Kerala, India, show no increase of cancer incidence when compared to their neighbours in low-level areas^{9,12}.

Studies on adaptive response and hormesis seem consistent with the old adage of “whatever doesn’t kill you makes you stronger”, but it is important to know that radiation, like most other high-dose toxins, is a part of our everyday life. North Americans receive on average 2-3mGy/year from natural sources, so this raises two questions. First, how much of a protective effect are we already subject to from the priming nature of background dose? And secondly (and relevant to the topic of this thesis), what happens if we take this exposure away – does the adaptive response logic hold true in reverse? The next chapter discusses how androgen levels confer an inherent resistance to radiation, and the effects produced by their removal.

Androgen Deprivation Therapy

Androgen deprivation therapy (ADT) is a technique by which testosterone levels are depleted by means of surgical or chemical castration. ADT has been used independently as an effective means of curbing growth of metastatic prostate cancer since 1941²⁵, and radiotherapy has shown success in treating localized prostate tumours^{26,27}. However there is low consistency in degree of response to radiotherapy, and in approximately 45% of cases, radiotherapy fails when given alone²⁷. Attempts to increase tumour control by increasing total dose given were successful, but also resulted in increased toxicity to the rectum²⁸. With the highest degree of radiotherapeutic precision available already being employed, another approach was necessary.

Studies using ADT and radiotherapy as adjuvant therapies to one another have shown increased local tumour control, with five-year disease-specific failure rates decreasing by 45%²⁹ – 71%³⁰, consistent with a 40% improvement at 10 years³¹.

Similar improvements were found with the ADT-radiotherapy combined treatment over ADT alone, with 10 year disease-specific failure reduction of 43%³² - 50%³³. Effects of both treatment options on quality of life did not differ significantly³⁴, and the American Society of Clinical Oncology is now suggesting that this combined modality may become the new standard of care³².

Until recently, the mechanism for this effect was unclear. However, a 2007 study by Pinthus et al. has shown that a possible explanation for the effect relates to testosterone inducing an adaptive response to oxidative stress. It was shown that testosterone provoked a dose-dependent increase in ROS production in 22rv1 human prostate cancer cells *in vitro*, as well as improved survivability to acute exposures of hydrogen peroxide and radiation. Cells exposed to testosterone also showed significant upregulation in oxidative defence enzymes manganese superoxide dismutase (MnSOD), clusterin, and catalase when compared to cells grown in androgen-deficient environments. The effect was also producible *in vivo* with human prostate cancer xenografts grown in SCID mice showing elevated levels of oxidative stress marker 8-OHdG in mice transplanted with 10mg slow-release testosterone as compared those that had been castrated⁷.

While this was the first study to explain the radiosensitizing effects of androgen deprivation, the effects of androgens on the oxidative environment have been known since 1997. It was shown that androgen levels were capable of increasing oxidative stress in LNCaP prostate cancer cells, as well as glutathione levels. The effect was ascribed to altered mitochondrial activity³⁵.

Since then, understanding of ROS-producing NADPH oxidases, which will be explained in detail in the next chapter, have expanded dramatically. A follow-up to the 2007 Pinthus, *et al.* study was published in 2010 that provided evidence for testosterone-mediated ROS upregulation being attributable to NADPH oxidase stimulation and increased expression of important NADPH oxidase subunits³⁶.

The radiosensitizing properties of ADT may be explained by the adaptive response mechanism. Naturally high levels of ROS production stimulate prostate tissue to generate equally high levels of oxidative defence enzymes, such that the tissue has a significantly increased ability to mitigate the ROS produced during irradiation with radiotherapeutic intentions. By decreasing testosterone by surgical or chemical castration, this protective adaptation can be eliminated, and patients can be more successfully treated with radiation therapy.

ADT is a proven method of sensitizing prostate tumours to radiation, and this combined modality appears to be on its way to becoming the gold standard. However, with the mechanism now believed to be related to the effects of testosterone on NADPH oxidase expression and activity, it becomes another challenge to improve the therapy.

NADPH Oxidase (NOX) & NOX-inhibition by Apocynin

NADPH Oxidases (nicotinamide adenine dinucleotide phosphate-oxidases) are a family of proteins found in the plasma membrane that transport electrons either into or out of the cell, resulting in the generation of free radicals or reactive oxygen species (ROS). The five main isoforms are NOX1, NOX2, NOX3, NOX4, and NOX5, DUOX1 and DUOX2, all of which generate superoxide by the transfer of an electron from NADPH to a dioxygen molecule, forming NADP⁺ and superoxide, which quickly dismutates into hydrogen peroxide and other reactive oxygen species^{37,38}. The catabolism of NADPH is important in many functions, producing ROS for signalling purposes or for the respiratory burst whereby cells release large amounts of ROS as an antibacterial mechanism.³⁹ The role of reactive oxygen species is very diverse, as are its adverse effects, as levels of ROS production have been positively linked to diabetes, congestive heart failure, Alzheimer's disease, alcoholic liver disease, and cancer^{2,40,41,42,43,44, 45}.

ROS are generated by other functions as well, such as mitochondrial generation of ATP⁴⁶, however NADPH oxidation typically accounts for a major fraction of ROS production. NADPH oxidation was the first identified process that appears to generate ROS as its primary response, rather than a byproduct³⁸. Indeed, cellular systems with higher NADPH oxidase concentrations also show heightened capacity for generating ROS⁴⁷. While all NADPH oxidase isoforms generate ROS, the first identified and most heavily-studied NOX2 isoform, also known as the ‘phagocyte NADPH oxidase’ has been found to be the primary isoform correlating NADPH oxidase concentration and superoxide generation^{38, 48}. One study showed that an eight-fold increase in NOX2 expression upregulated the production of superoxide by two- to three-fold on its own⁴⁹.

NOX2, also referred to as gp91-phox is very widely distributed in mammalian tissues. It is found in B lymphocytes, skeletal and smooth muscle cells, prostate tissue, among many other tissues⁵⁰. It is also known as cytochrome b558 component A (or CYBA).

Cytochrome b558 component B (CYBB) is also an important NADPH oxidase subunit called p22-phox, which is a fundamental component to the complete formation of NOX1, NOX2⁵¹, NOX3, and NOX4³⁸. CYBA and CYBB exist in cytochrome b558 in equal concentration, making p22-phox a convenient subunit to measure as an indication of NOX2 upregulation as well⁵².

As previously mentioned, androgen-deprivation is one effective means of NADPH oxidase inhibition in androgen-regulated tissues like the prostate, however NADPH oxidase may also be inhibited by chemicals aptly referred to as NADPH oxidase

inhibitors. One such inhibitor is apocynin (also known as acetovanillone, or 4-hydroxy-3-methoxyacetophenone), a chemical derived from the plant *Picrorhiza kurroa*⁵³ (see Figure 1). Apocynin inhibits the release of superoxide by NADPH oxidase by blocking migration of p47phox to the cell membrane in NOX2^{49, 53, 54}, and has been successfully employed in many studies to decrease or prevent the generation of ROS^{36, 55, 56, 57}. It has also been suggested that apocynin derivatives can act as a scavenger for superoxide ions, thereby directly decreasing ROS concentration as well as inhibiting its production⁵⁵. The chemical structure of apocynin is shown in Figure 1.

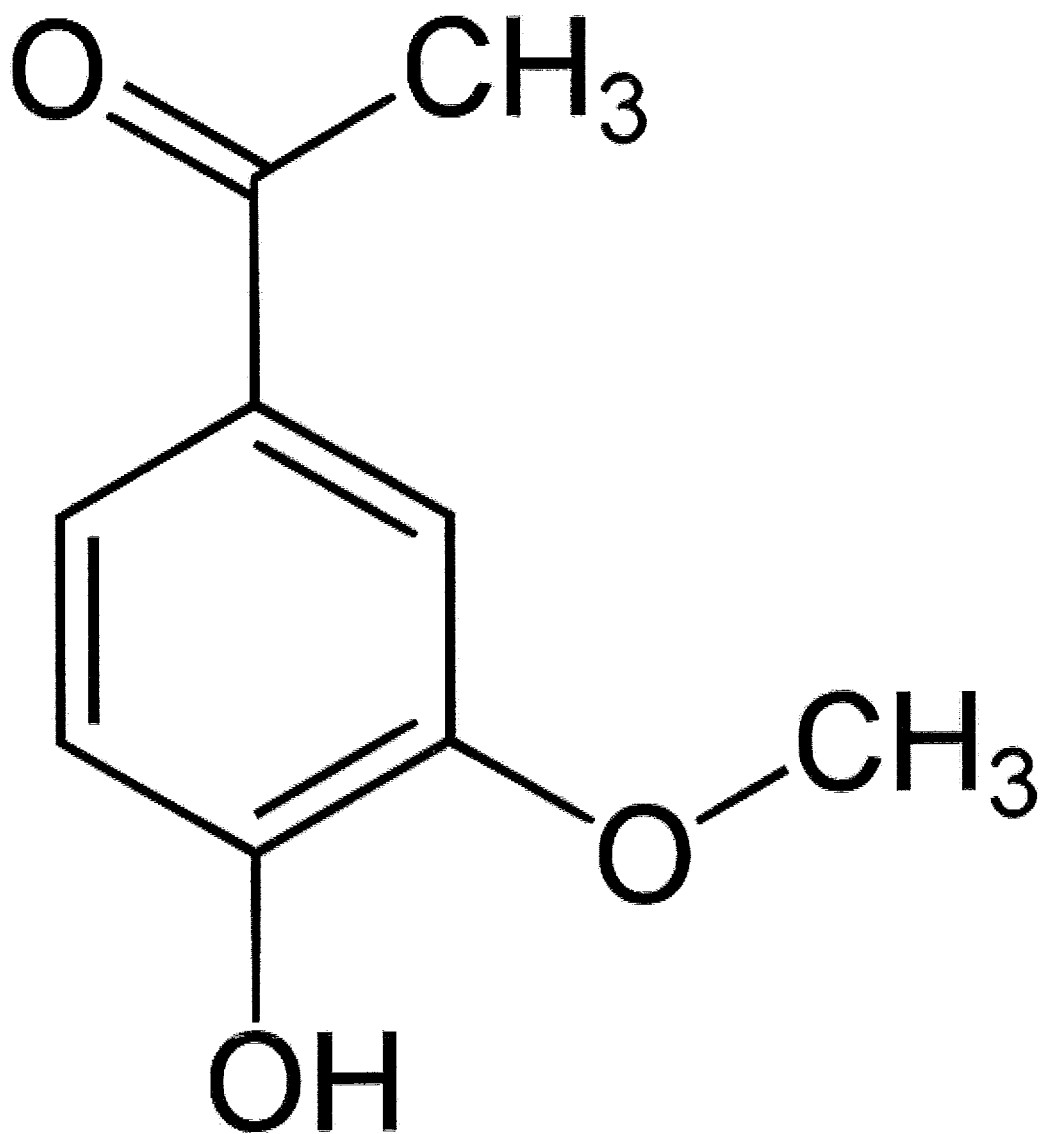


Figure 1 - Chemical Structure of Apocynin

In this form, apocynin does not have an inhibitory effect on ROS production by NADPH oxidase^{53, 58,59,60}. In fact, one study has shown that apocynin stimulated ROS production in non-phagocytic cells⁶¹, as well as another that showed increased H₂O₂, malonyldialdehyde and lactate dehydrogenase⁶². However, when apocynin is 'activated', or formed into diapocynin by interaction with peroxidase, it then functions effectively as an NADPH oxidase inhibitor. In vascular cells, such as lymphocytes, myeloperoxidase (MPO) interaction results in the formation of apocynin dimers. Cell types differ in their basal levels of MPO, and this has a profound effect on the portion of apocynin that becomes activated⁵⁵. This effect has been artificially reproduced many times via activation with horseradish peroxidase^{53, 61} and soybean peroxidase^{59,60}.

Without artificial activation, apocynin could then have zero or potentially opposite effects in different cell lines *in vitro*. However, the effect likely occurs naturally during metabolism *in vivo*⁶³, and many studies have shown ingested apocynin to successfully inhibit ROS^{64,65,66,67}

Hypothesis and Objectives

Hypothesis:

NADPH oxidase inhibition by apocynin results in decreased levels of basal ROS production in prostate cancer cells and renders them more sensitive to radiation treatment.

Objective:

1. Determine the effects of apocynin on radiosensitization of MATLyLu cells *in vivo* in Cop/Hsd rats, measured by survival time post-injection and rate of growth
2. Verify that any observed effects can be explained by NADPH oxidase inhibition *in vivo*
3. Determine that MATLyLu cells generate ROS by NADPH oxidase activity
4. Determine the effects of apocynin on NADPH oxidase inhibition, ROS production, and radiosensitization in MATLyLu cells *in vitro*
5. Determine the different affects of apocynin and artificially-synthesized diapocynin on NADPH oxidase activity, ROS production and radiosensitivity in MATLyLu cells *in vitro*.

It is well documented in the literature that apocynin inhibits NADPH oxidase activity in many cell lines, and that this inhibition has resulted in increased radiosensitivity in human prostate cancer cells. To determine its clinical feasibility, the possibility for sensitizing syngeneic tumours via oral or intraperitoneal administration must be explored. Therefore the first objective was achieved by designing experimental groups to test the effects of route of administration as well as timing relative to radiation treatment on the response of MATLyLu rat prostate cancer cells in male Cop/Hsd rats. The seven treatment groups are listed in Table 1.

Verification of NADPH oxidase inhibition *in vivo* (objective two) was tested by measuring levels of NADPH oxidase subunit expression in tumour samples *ex vivo*.

A proposed mechanism for apocynin radiosensitization is the ability of apocynin to inhibit NADPH oxidase activity, which results in a decrease in basal ROS production and in turn decreased antioxidant defence capability. The second and third objectives were met by verifying NADPH oxidase activity in MATLyLu cells, attempting its inhibition with the NADPH oxidase inhibitor apocynin, and observing changes in radiosensitivity.

There is some indication that apocynin must be synthesized into diapocynin to achieve its NADPH oxidase inhibitory effect, and that this synthesis may occur differently *in vitro* than *in vivo*, and that the degree to which cells can autsynthesize apocynin into its dimer form varies widely. Therefore a fifth set of experiments were designed to test the objective of identifying differences between inactivated apocynin and diapocynin. This was attempted by comparing inhibition of ROS production and radiosensitization.

Experiments for objectives two through five are listed in Table 2.

Table 1 - Groups for *in vivo* study; 20 or 21 180-220g male Cop/Hsd rats per group. Group naming was used in data collection only; groups are described in full detail in all analyses.

Group	Apocynin	Radiation
CA	None	None
CB	None	10Gy/5fx/5days
CC	5mM PO in drinking water x 14 days (5 days before radiation treatment, 5 days during radiation treatment, and 4 days after radiation treatment)	None
CD	5mg/kg IP injections x 14 days (5 days before radiation treatment, 5 days during radiation treatment, and 4 days after radiation treatment)	None
EA	5mM PO in drinking water x 14 days (5 days before radiation treatment, 5 days during radiation treatment, and 4 days after radiation treatment)	10Gy/5fx/5days
EB	5mg/kg IP injections x 14 days (5 days before radiation treatment, 5 days during radiation treatment, and 4 days after radiation treatment)	10Gy/5fx/5days
EC	5mm PO in drinking water x 5 days prior to day 1 of radiation treatment	10Gy/5fx/5days

Table 2 - Experiments to accomplish objectives two through five

Objective	Method
Attribute radiosensitizing effect to alterations of NADPH-oxidase subunit expression	Western Blot of tumours <i>ex vivo</i>
Confirm NADPH oxidase activity in MATLyLu cells <i>in vitro</i>	RT-PCR (reverse transcriptase polymerase chain reduction)
Determine effects of apocynin on ROS production <i>in vitro</i>	NBT Assay with WST-1 assay to normalize ROS production as a per-cell function
Determine effects of apocynin on radiosensitization <i>in vitro</i>	Colony forming assay and cell proliferation assay
Determine effects of diapocynin on ROS production <i>in vitro</i>	NBT Assay with WST-1 assay to normalize ROS production as a per-cell function
Determine effects of diapocynin on radiosensitization <i>in vitro</i>	Colony forming assay and cell proliferation assay

Materials and Methods

2.1 MATLyLu Standard Growth Curves

The MATLyLu rat prostate cancer cell line was acquired from Dr. Jehonathan Pinthus's lab at the Juravinski Cancer Center (Hamilton, ON) and cultured at 37°C in a humidified atmosphere under 5% CO₂ in air. The MATLyLu culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) with L-Glutamine, pyridoxine and pyruvate, and 4.5g/K glucose (VWR, Mississauga, ON), 10% fetal bovine serum (VWR, Mississauga), and 1% penicillin/streptomycin (VWR, Mississauga).

Upon receiving the MATLyLu cell line from Dr. Pinthus' lab, large quantities were quickly grown and snap-frozen in freezing medium (10% dimethyl sulfoxide (DMSO) (VWR, Mississauga, ON) in complete media with 20% FBS) in liquid nitrogen for future retrieval and use.

2.1.1 Effect of Passage Number and Trypsinization Frequency on MATLyLu Cell Growth

Two cryovials of MATLyLu cells, A and Z, were thawed and plated in a pair of vented, bent-neck T75 culture flask in 25mL complete media at 80,000 cells/mL. Flask A was allowed to grow for 48 hours, and was then washed twice with 1X PBS and trypsinized (0.05%/0.053mM EDTA in HBS, Fisher Scientific, Burlington, ON), and was then diluted 1:2 and passaged into Flask B, which was then allowed to grow for 48 hours before being diluted 1:2 and passaged into Flask C, and so forth up until F. Flask Z was treated identically but was allowed 96 hours as an initial growth period before entering the same 48-hour incubation-and-trypsinization pattern as flask A into flask Y, X, W, and V. Each plating involved a second flask that was counted after 24 hours, such that a count was available for every day. Comparisons were later made to identify differences in growth tendencies between passage numbers and to see if a longer initial growth period was of benefit.

Three other flasks, TRYPX-1, TRYPX-2, and TRYPX-3 were also plated in 25mL complete media at 80,000 cells/mL and allowed to grow for 1 day, 2 days, or 3 days (as indicated by sample name) between trypsinization. The process went on for 12 days.

2.1.2 Calibration of Developed MATLyLu Growth Protocol

From the trials in 2.1.1, a protocol was developed such that one cryovial of MATLyLu cells was plated in 25mL complete media at 80,000 cells/mL and

incubated for 24 hours, following which the media was replaced, and the culture was incubated for 72 hours further. At this 96-hour point, cultures were trypsinized as previous described and diluted 1:25 and passaged into a new flask and incubated for 72 hours once again. Cell counts were taken at the 96-hour point and 168-hour point four times over a four week period and analyzed for consistency in section 3.1.2.

2.2 MATLyLu Tumour Growth in Rats *in vivo*: Pilot Trials

MATLyLu cells were grown by the method outlined in section 2.1.2 and drawn up into tuberculin syringes with 23 gauge needles in 0.1mL volumes at a concentration of 5×10^6 cells/mL, such that each syringe held 5×10^5 cells. Rats were anaesthetized with 5% isoflurane at 3L/min oxygen, had their hind limbs shaved and disinfected with Webcol wipes, and were given one injection subcutaneously on the hind limb (some unilaterally, some bilaterally, as outlined below in each trial). The rats were then monitored for tumour growth three times weekly until tumours reached a pre-identified endpoint, such as ulceration, poor body condition, or size (outlined below), before being euthanized.

2.2.1 MATLyLu Tumour Growth in Cop/Hsd Rats *in vivo* with 10mm diameter endpoint

Three 150-200g (2-3 months old) male Cop/Hsd “Copenhagen” rats were acquired from Harlan Laboratories (San Diego, CA). Two were injected unilaterally with MATLyLu cells as described above, and one was injected bilaterally. They were monitored until they developed tumours with a maximum diameter of 10mm or larger and were then euthanized.

2.2.2 MATLyLu Tumour Growth in F-344 Rats *in vivo* with 10mm diameter endpoint

Three 150-200g (2-3 months old) male F344 Fischer/Copenhagen rats were acquired from Harlan Laboratories (San Diego, CA). Each was injected bilaterally with MATLyLu cells as described above. They were monitored until they developed tumours with a maximum diameter of 10mm or larger and were then euthanized.

2.2.3 MATLyLu Tumour Growth in Cop/Hsd Rats *in vivo* with 35mm diameter endpoint

Ten 150-200g (2-3 months old) male Cop/Hsd “Copenhagen” rats were acquired from Harlan Laboratories (San Diego, CA). Two were injected unilaterally with MATLyLu cells as described above, and one was injected bilaterally. They were monitored until they developed tumours with a modified maximum diameter of 35mm or larger and were then euthanized.

2.3 Design and Dosimetry of Rat Irradiator

Cop/Hsd rat carcasses were collected from pilot studies and used to develop an irradiation chamber to be used to isolate radiation treatment to the affected hind limb. The design was developed in collaboration with Yosh Kitamura at McMaster University. The chamber was designed around the largest rat collected, and given and internal size of 12cm x 23cm x 5cm. Three holes were drilled in the front for airflow and anaesthetic tubing. The unshielded portion would be over the right hind limb only and would have an area of 4.5cm x 10.5cm. Tail and spinal cord would be kept out of the field at all times. The remainder of the chamber would be covered with removable lead blocks during

irradiation, notched at their meeting points to prevent leakage (Figure 4). The thickness of lead was calculated in order to bring whole body dose to below 10mGy over the course of 10Gy treatment, or a 1000-fold reduction in dose. To find the number of half-value layers required, the following formula was employed:

$$\text{Equation 1: } \frac{1}{1000} = \frac{1}{2^n}$$

Therefore,

$$\text{Equation 2: } n = \frac{\log 1000}{\log 2} = 9.96 \cong 10$$

Where n is the number of half value layers required.

The half-value layer for 0.662keV γ -rays attenuated by lead is 0.65cm, therefore the lead shield was designed to a thickness of 6.5cm. A 6.5cm thick slab of pure lead was purchased from Alchemy Extrusions (Hamilton, ON) and processed into three stackable blocks by Yosh Kitamura.

Figure 2 and Figure 3 show the design of the irradiator through to its construction.

Following irradiator construction, its shielding properties had to be verified, and the dosimetry across the open field measured for proper positioning of the rats during irradiation. A Farmer Dosimeter ionization chamber was used to measure the exposure (in Roentgens) at various points inside the irradiator, including a 0.5cm x 0.5cm matrix inside the open field. A 10-second exposure was given using the Taylor Radiobiology Room cesium-137 source at McMaster University for each point at 26cm from the source, which is approximately 4 Roentgens, using the provided dose-rate calibration curves, and adjusting for f-factor rad-Roentgen adjustment of $\frac{1}{0.873}$ Roentgen/rad.

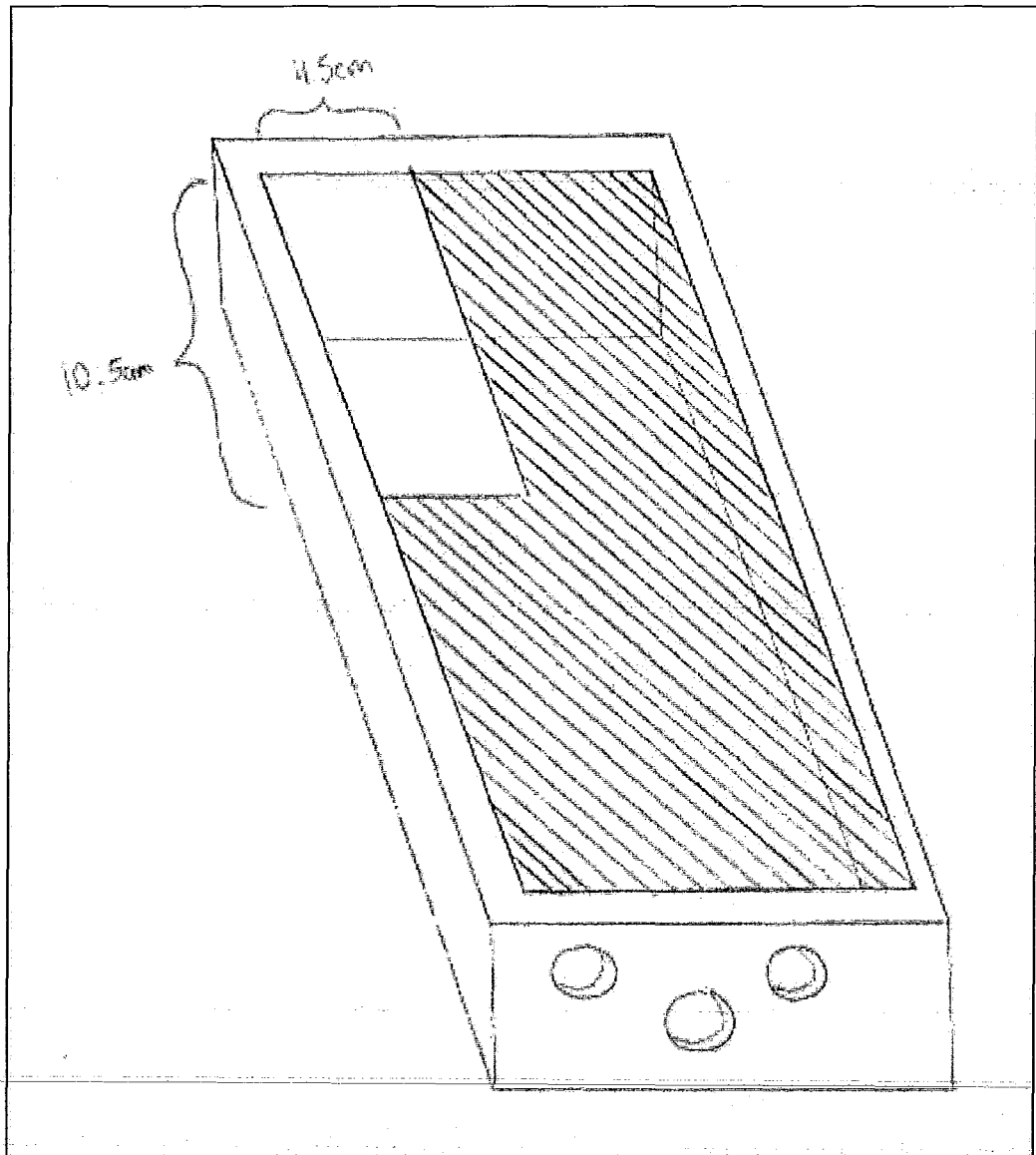


Figure 2 - Irradiator Design. The black hashed area indicates the required lead shielding.

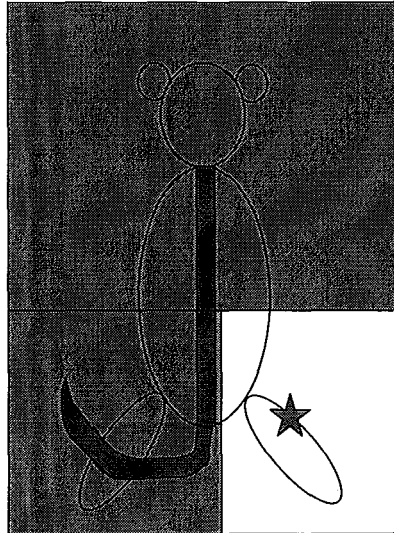


Figure 3 - Irradiator was designed such that spinal cord tissue would be entirely shielded, and the only object of interest in the open field is the tumour, indicated by the star

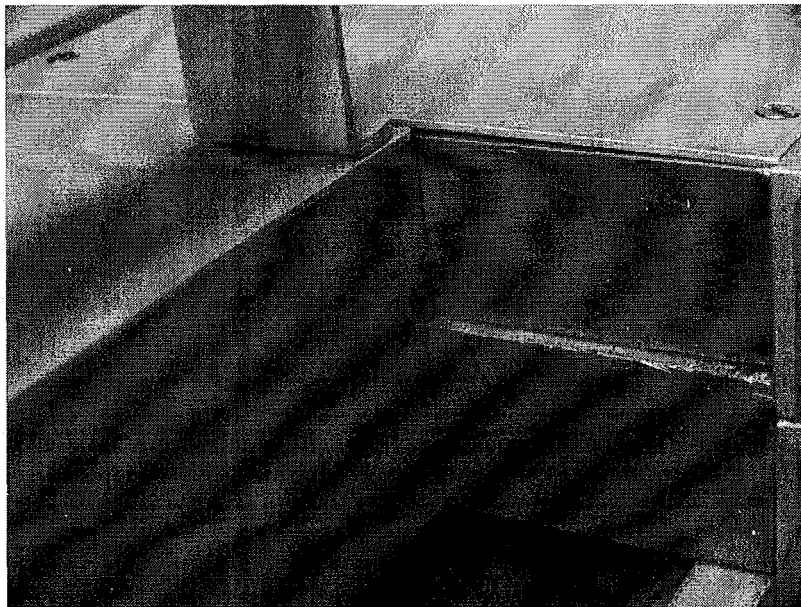


Figure 4 - Lead blocks were notched at points of overlap to prevent leakage through the shield

2.4 Effects of Apocynin on Radiosensitization *In Vivo*

Seven groups of twenty 180-220g male Cop/Hsd “Copenhagen” rats were used to determine the effects of apocynin on prostate tumour radiosensitivity *in vivo*. All groups were injected bilaterally with 5×10^5 MATLyLu cells as per methods outlined in section 2.2 and monitored daily Monday-Friday for growth. Tumour size was measured with calipers, with the largest diameter for each tumour being the recorded value. On day 11 post-injection, each rat underwent a biopsy procedure under sterile conditions and 3% isoflurane anaesthetic at 2L/min oxygen. The tumour on the left side of the rat tumour was completely removed and stored half in formalin and half snap-frozen in liquid nitrogen. A skin sample and the left submandibular salivary gland were also collected for future studies and similarly stored. Leg incision was sutured with sterile 4-0 vicryl ties and continuous sutures along the inner layers of the skin. The exterior skin was sealed with Vetbond glue. The incision in the submandibular region was repaired using sterile staples. Apocynin and radiation administration was staggered throughout the groups as initially indicated in Table 1. A summary of the apocynin and radiation administration by group was as follows: Two groups received apocynin (Sigma, Burlington, ON) in their drinking water at 5mM for 14 days, beginning on day 9 (one which later received radiation, one which did not). Another group received apocynin in their drinking water at 5mM for 5 days, beginning on day 9, in addition to radiation. Two groups received apocynin via intraperitoneal injection at 5mg/kg daily for 14 days, beginning on day 9 (one which later received radiation, one which did not). One group received radiation and

no apocynin, and one group had no treatment at all. All radiation treatments were performed under 3% isoflurane anaesthesia to a total of 10Gy of 0.662keV γ -radiation to the right hind limb over 5 daily 2Gy fractions, beginning on day 14 post-injection. Full details of the treatment schedules can be found in Table 1. Full details of the biopsy procedure, apocynin preparation and administration, and radiation therapy administration can be found in the Appendix.

All rats in all groups were monitored daily for tumour size and health. Endpoints were defined as a tumour exceeding 35mm in any dimension, an ulcerated tumour, poor body condition or obvious signs of distress. Euthanasia was carried at any endpoint by CO₂ suffocation.

2.4.1 Survival Time

Groups were compared for survival time (in days post-injection) among rats who reached endpoint by either right-sided tumour diameter exceeding 35mm or right-sided tumour ulceration. Comparison was done by employing Kaplan-Meier survival curves and the log-rank test. Results are available in section 2.4.1.

2.4.2 Tumour Growth

Tumour size was measured daily for every rat Monday-Friday. Comparisons were made between all groups (excluding outliers and animals which did not develop any tumour) using a Chi-squared test in section 2.4.2.

2.4.3 Doubling Time

Doubling time was calculated for groups by modeling exponential growth for each rat from day 14 until endpoint. Modelling was done using the GROWTH function in Microsoft Excel, which models exponential growth based on data from individual animals. The GROWTH function returns a projected tumour volume for a given day. For example, for day 22 with rat CA1, GROWTH returns a projected diameter of 27.7mm. The actual diameter is 28mm.

Doubling time was calculated from Equation 3, which would predict exponential growth,

$$\text{Equation 3: } V = V_0 e^{\lambda t}$$

Upon rearranging and choosing subsequent days (such that $t=1$),

$$\text{Equation 4: } \lambda = \ln \left(\frac{V}{V_0} \right)$$

Equation 3 can also be rearranged to find doubling time, t_{dbl} , by assuming a value for V twice that of V_0 ,

$$\text{Equation 5: } t_{dbl} = \frac{\ln 2}{\lambda}$$

Therefore,

$$\text{Equation 6: } t_{dbl} = \frac{\ln 2}{\ln \frac{V}{V_0}}$$

Where V_0 is the volume first of subsequent days and V is the second. The $\ln$ of $\frac{V}{V_0}$ ratio will be the same throughout the growth model. Days 24 and 25 were used in all calculations.

2.5 Measurement of NADPH Oxidase Function in MATLyLu Cells *In Vitro*

Real-Time Polymerase Chain Reaction (RT-PCR) was carried out on MATLyLu cells to verify gene expression for the NOX subunits NOX1, NOX2, NOX3 and NOX4 by Zhefeng Hou at the laboratory of Dr. Jehonathan Pinthus at the Juravinski Cancer Centre (Hamilton, ON) by the following methods which have been successfully employed previously by Peng, et al.⁶⁸:

Real-time RT-PCR was performed using a MiniOpticon system (Bio-Rad Laboratories) with SYBR GreenER two-step qRT-PCR kit (#11764 –100, Invitrogen). Briefly, RNA was extracted from MATLyLu cells using TRIZOL and was reverse transcribed using superscript III reverse transcriptase. Primer sequences for real time RT-PCR amplification were as follows:

NOX1: (forward) CACTGTGGCTTTGGTTCTA and (reverse)

TGAGGACTCCTGCAACTCCT (Size 240, Gene Bank #NM_053683)

NOX2: (forward) GTGGAGTGGTGTGAATGC and (reverse)

TTTGGTGGAGGATGTGATGA (Size 219, Gene Bank # NM_023965);

NOX3: (forward) GACCCAACTGGAATGAGGAA and (reverse)

AATGAACGACCCTAGGATCT (Size 150, Gene Bank # NM_001004216);

NOX4: (forward) CGGGGTGGCTTGTGAAGTAT and (reverse)

CGGGGTGGCTTGTGAAGTAT (Size 205, Gene Bank # NM_053524).

Relative RNA quantification was calculated using the comparative threshold (CT) method using the formula “ $2^{-\Delta CT}$ ” where ΔCT is the difference between the threshold cycle of the

given target cDNA between normoxia and CIH. The C_T value was taken as a fractional cycle number at which the emitted fluorescence of the sample passes a fixed threshold above the baseline. Values were compared with an internal standard gene actin.

RT-PCR was performed by Zhefeng Hou at Dr. Jehonathan Pinthus's lab at the Juravinski Cancer Centre (Hamilton, ON)

2.6 Effects of Apocynin on ROS Production in MATLyLu Cells *In Vitro*

ROS production was measured using the nitroblue tetrazol (NBT) (Sigma, Burlington, ON) assay. For this Assay, MATLyLu cells are seeded into a 96-well plate in 200 μ L aliquots at 25000 cells/mL and grown for 48 hours. 4 or 24 hours before beginning the NBT assay, apocynin was added at 200 μ M or 1000 μ M. One row of wells was left empty for controls/blanks on the reader. NBT (Sigma, Burlington, ON) was dissolved in methanol (20mg in 200 μ L) and diluted in 9.8mL PBS. The media was then removed from the plate and 60 μ L NBT solution was added to each well before incubating the plate at 37°C for 1.5 hours. Following incubation, excess NBT solution is removed from the plate, the wells are washed twice with PBS, and 70 μ L 2M KOH and 80 μ L DMSO were added to each well, forming blue crystals where the NBT was taken up by the cells. The plate was then put on a sonicator for 10 minutes at 60 sonics, or until all crystals have broken down, and then it was read by spectroscopy at 620nm.

2.7 Effects of Apocynin on Cell Proliferation in MATLyLu Cells *In Vitro*

Cell proliferation was measured using the WST-1 tetrazolium salt assay. MATLyLu cells were plated into a 96-well plate in 200 μ L aliquots at 5000 cells/mL and incubated. 24 hours later, apocynin was added at 100 μ M, 200 μ M, 500 μ M, 1000 μ M, and 2000 μ M.

Media was removed and replaced with phenol red-free RPMI 1640 (VWR, Mississauga, ON) containing 10% FBS, 2mM L-glutamine, 1mM Na-pyruvate (VWR, Mississauga, ON), and 50µM mercaptoethanol with 1:10 WST-1 reagent (Roche, Mississauga, ON) at 100µL per well and incubated for 1 hour. The plate was then read by spectroscopy at 620nm.

2.8 Effects of Apocynin on MATLyLu Survival Post-Irradiation *In Vitro*

Three different methods were employed to measure to post-irradiation of MATLyLu cells treated or untreated with apocynin. They are as follows:

Method A: Measurement of Cell Proliferation Following Irradiation

MATLyLu cells were grown in complete media and were given apocynin at either 200µM or 1000µM for either 4, 24, or 72 hours before irradiation. Cells underwent irradiation on ice at 0, 2, 4, 6, 8, or 10Gy, in the same T75 or T25 flasks they were grown in, and immediately trypsinized and split afterwards in either duplicate or quadruplicate at equal concentrations as calibrated via centrifugation and dilution. Some flasks also received apocynin at the time of splitting (after irradiation) to test the effects of apocynin in the DNA repair process after the immediate insult. Cells were allowed to grow for 96 hours before being resuspended and counted on our Z2 Coulter Cell Counter.

Method B: Measurement of Colony Formation Following Irradiation

MATLyLu cells were grown in complete media and given apocynin at either 200µM or 1000µM for 24 hours prior to irradiation. Cells underwent irradiation on ice at 0, 1, 2, or

3Gy, in the same T75 or T25 flasks they were grown in, and were immediately trypsinized and diluted such that 100 cells could be seeded into slide-flasks with 2.5mL complete media, in quadruplicate. Cells were allowed to grow for 168 hours, after which they were fixed with 1:3 ethanol in acetic acid fix solution, allowed to dry for 24 hours, and then stained with 10% giemsa stain (Sigma, Burlington, ON) 24 hours after staining, the colonies were counted under microscope, although most were visible to the eye. A viable colony was defined as a colony of 50 or more cells.

Method C: Measurement of Colony Formation Following Irradiation, Alternate Method

MATLyLu cells were grown in complete media then plated at 200 cells/T12.5 flask in 4mL complete media in quadruplicate. 24 hours later, media was replaced and apocynin added at 1000 μ M for 24 hours prior to irradiation. Cells underwent irradiation on ice at either 0 or 2Gy and then incubated. Cells were incubated for 168 hours, after which they were fixed with 1:3 ethanol in acetic acid fix solution, allowed to dry for 24 hours, and then stained with 10% giemsa stain (Sigma, Burlington, ON). 24 hours after staining, the colonies were counted under microscope, although most were visible to the eye. A viable colony was defined as a colony of 50 or more cells.

2.9 Effects of Apocynin on NADPH Oxidase Expression in MATLyLu Tumours in Cop/Hsd Rats *Ex Vivo*

Protein lysate solutions were made from rat tissue samples preserved in formalin by snap-freezing 0.1g tissue with dry ice and grinding it into a powder using a pre-cooled mortar and pestle. 600 μ L lysate buffer was added to each sample, and was then processed into a

solution with TissueRuptor for 30s and allowed to sit on ice for 10 minutes, following which 3X Laemmi buffer was added to the solution to 33% concentration. Solutions were then sonicated three times for 10s and then centrifuged for 10 minutes to separate proteins from pellet. Pellet was discarded and the proteins were all equalized to 2 $\mu\text{g}/\mu\text{L}$. Lysates were run via gel electrophoresis through upper and lower acrylamide gel at 45V for 1hr and 100V for 1hr, respectively. The gel was then placed on a membrane pre-soaked in methanol in 1X transfer buffer, sandwiched between two pieces of filter paper and a sponge on each side, and transferred at 85V for 1hr. The membrane was then washed in 1X TBS-T for 5min before being blocked in 5% milk for 1hr. The membrane was then washed three times more in 1X TBS-T for 5 minutes each, and then shaken in 8mL 2.5% milk with 80 μL p22-phox antibody (Santa Cruz) or 8 μL actin antibody (Santa Cruz) at 4 degrees Celsius overnight. The next morning, membranes were again washed for 5 minutes in 1X TBS-T three times and were then shaken in 8mL 2.5% milk loaded with 8 μL goat anti-rabbit antibody (Santa Cruz) for p22-phox, or 4 μL rabbit anti-mouse antibody (Santa Cruz) for actin at room temperature for 1hr. Membranes were washed thrice more in 1X TBS-T for five minutes and then soaked in Super Signal (source needed) for two minutes before being brought to a dark room and exposed to film for 2-20s (time determined by trial runs on-site). Films were developed in Bio-Rad developer (Thermo-Fisher, Burlington, ON) and bands were quantified on Bio-Rad Molecular Imager Gel Doc System with Quantity One 1-D Analysis Software. Statistical comparisons were made by comparing the average values of band darkness, as well as the peak (most saturated) values of band darkness.

2.10 Synthesis of Diapocynin

2g of apocynin was stirred into in 200mL of boiling deionized water. 0.15g iron(II) sulphate heptahydrate and 1.6g of sodium peroxydisulfide were added and stirred for five minutes. A brown precipitate formed. The solution was left to cool at room temperature and was then filtered. The collected precipitate was dissolved in 25mL 3M NaOH and then reprecipitated by the addition of 15mL 6M HCl. The precipitate was filtered and washed three times with boiling deionized water and left in a dessicator overnight⁶⁸. Diapocynin prepared in this manner has been shown to be stable in DMSO at room temperature for 30 days.⁶⁹

Results

3.1 MATLyLu Standard Growth Curves

3.1.1 Effect of Passage Number and Trypsinization Frequency on MATLyLu Cell Growth

The standard growth curves were generated repeatedly over a two week period. Each data point on the curves represents the cell concentration from the culture medium of a single T25 flask. Curves with similar attributes are plotted together to identify differences created by variances in technique. Cell cultures that had been passaged once or twice seemed to display the highest and most consistent rate of growth. Cultures from passage 0 or greater than 2 did not seem to consistently grow as quickly. Frequency of trypsinization/dilution was tested at 24, 48, and 72 hours. Results did not generate consistently different growth trends between regimens of differing trypsinization frequencies.

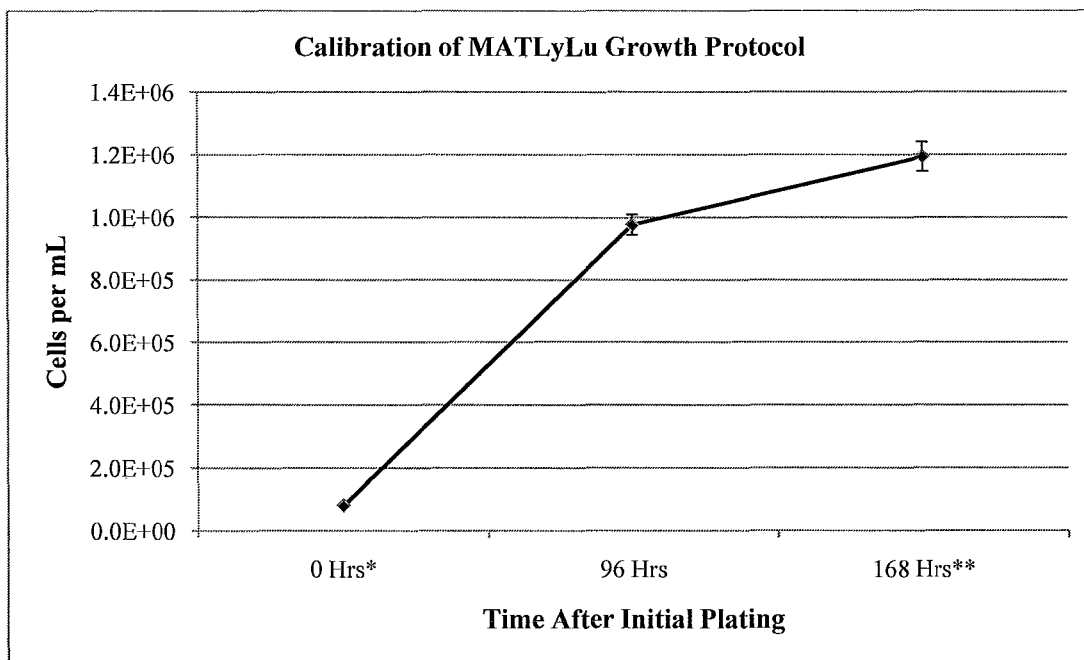
3.1.2 Calibration of Developed MATLyLu Growth Protocol

A protocol was developed based on these observations for growing MATLyLu cells for future experiments using 72 hour trypsinization periods, following an initial 24 hour settling period, and limiting growth to two passages before experimental use. Full details of this protocol are in section 2.1.2 and in Appendix C. This trial was repeated four times. The averaged cell counts and variances are listed in Table 3, and data is plotted with error bars are plotted in Figure 5.

Table 3 - Calibration of MATLyLu Growth Protocol

Time	Number of Cells per mL
0 Hours	80,000 +/- 0*
96 Hours	976,000 +/- 32,674
168 Hours**	1,193,500 +/- 46,340

Figure 5 - Calibration of MATLyLu Growth Protocol



* 2mL frozen MATLyLu cells at 1×10^6 /mL in 23mL complete media – value calculated and not counted

**Solution diluted 1:25 after 96 hours to prevent confluence from increasing beyond 100%

3.2 MATLyLu Tumour Growth in Rats *in vivo*: Pilot Trials

3.2.1 MATLyLu Tumour Growth in Cop/Hsd Rats *in vivo* with 10mm diameter endpoint.

Survival time was determined by following the progression of subcutaneously injected MATLyLu tumours into the hind limbs of three male Cop/Hsd rats until an endpoint diameter of 10mm was reached or exceeded and the rats were sacrificed. One rat was injected bilaterally in the hind limbs. The tumour size data is listed in Table 4. The survival time with variance is listed in Table 5.

3.2.2 MATLyLu Tumour Growth in F-344 Rats *in vivo* with 10mm diameter endpoint

Survival time was determined by following the progression of bilateral subcutaneously injected MATLyLu tumours in three male Fisher F-344 rats until an endpoint diameter of 10mm was reached or exceeded and the rats were sacrificed. No tumours developed in any Fisher F-344 rats over a period of 21 days.

3.2.3 MATLyLu Tumour Growth in Cop/Hsd Rats *in vivo* with 35mm diameter endpoint

Survival time was determined by following the progression of bilateral subcutaneously injected MATLyLu tumours in ten male Cop/Hsd rats until an endpoint diameter of 35mm was reached or exceeded and the rats were sacrificed. Average survival time was 17.3 days. The survival time with variance is listed in Table 6. The tumour size data is listed in Table 7 (D indicates diameter, V indicates volume) and plotted in Figure 6.

Table 4 - Tumour Growth Data for Pilot Trial in Cop/Hsd Rats with 10mm Diameter Endpoint

Day Post-Injection	Tumour Diameter (mm)			
	Rat A L Limb	Rat B L Limb	Rat C L Limb	Rat C R Limb
0.0	0.0	0.0	0.0	0.0
1.0	0.0	0.0	0.0	0.0
4.0	0.0	1.0	0.0	1.0
6.0	0.0	2.0	1.0	5.0
8.0	0.0	5.0	11.0	9.0
11.0	7.0	15.0	-	-
13.0	12.0	-	-	-

Table 5 - Survival Time for Pilot Trial in Cop/Hsd Rats with 10mm Diameter Tumour Endpoint

	Survival Time (days)
Rat A	13
Rat B	11
Rat C	8
Average	10.7 +/- 1.4

Table 6 - Survival Time for Pilot Trial in Cop/Hsd Rats with 35mm Diameter Tumour Endpoint

	Survival Time (Days)
Rat 1	16
Rat 2	21
Rat 3	No Tumour Developed
Rat 4	16
Rat 5	14
Rat 6	18
Rat 7	14
Rat 8	16
Rat 9	18
Rat 10	23
Average	17.3 +/- 1

Table 7 - Tumour Growth Data for Pilot Trial in Cop/Hsd Rats with 35mm Diameter Endpoint

Day	Rat 1-R		Rat 2-R		Rat 3-R		Rat 4-R		Rat 5-R		Rat 6-R		Rat 7-R		Rat 8-R		Rat 9-R		Rat 10-R	
	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7.0	1.0	0.5	2.0	4.2	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.5	1.0	0.5	2.0	4.2	0.0	0.0	2.0	4.2
9.0	3.0	14.1	5.0	65.4	0.0	0.0	4.0	33.5	0.0	0.0	2.0	4.2	5.0	65.4	5.0	65.4	1.0	0.5	6.0	113.1
11.0	9.0	381.7	11.0	696.9	0.0	0.0	7.0	179.6	0.0	0.0	4.0	33.5	8.0	268.1	9.0	381.7	2.0	4.2	6.0	113.1
14.0	14.0	1436.8	17.0	2572.4	0.0	0.0	19.0	3591.4	0.0	0.0	9.0	381.7	20.0	4188.8	16.0	2144.7	8.0	268.1	13.0	1150.3
16.0	18.0	3053.6	23.0	6370.6	0.0	0.0	25.0	8181.2		0.0	12.0	904.8			22.0	5575.3	12.0	904.8	18.0	3053.6
18.0		0.0	26.0	9202.8	0.0	0.0				0.0	18.0	3053.6					17.0	2572.4	21.0	4849.0
21.0		0.0	35.0	22449.3	0.0	0.0													28.0	11494.0
23.0		0.0			0.0	0.0													35.0	22449.3

Day	Rat 1-L		Rat 2-L		Rat 3-L		Rat 4-L		Rat 5-L		Rat 6-L		Rat 7-L		Rat 8-L		Rat 9-L		Rat 10-L	
	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	4.2	3.0	14.1	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.5	0.0	0.0
9.0	1.0	0.5	0.0	0.0	0.0	0.0	8.0	268.1	8.0	268.1	0.0	0.0	0.0	0.0	1.0	0.5	5.0	65.4	0.0	0.0
11.0	5.0	65.4	3.0	14.1	0.0	0.0	12.0	904.8	12.0	904.8	0.0	0.0	5.0	65.4	4.0	33.5	8.0	268.1	4.0	33.5
14.0	14.0	1436.8	9.0	381.7	0.0	0.0	17.0	2572.4	19.0	3591.4	0.0	0.0	8.0	268.1	25.0	8181.2	13.0	1150.3	9.0	381.7
16.0	17.0	2572.4	14.0	1436.8	0.0	0.0	19.0	3591.4			0.0	0.0			29.0	12770.1	18.0	3053.6	12.0	904.8
18.0			16.0	2144.7	0.0	0.0					0.0	0.0					21.0	4849.0	15.0	1767.1
21.0			24.0	7238.2	0.0	0.0													21.0	4849.0

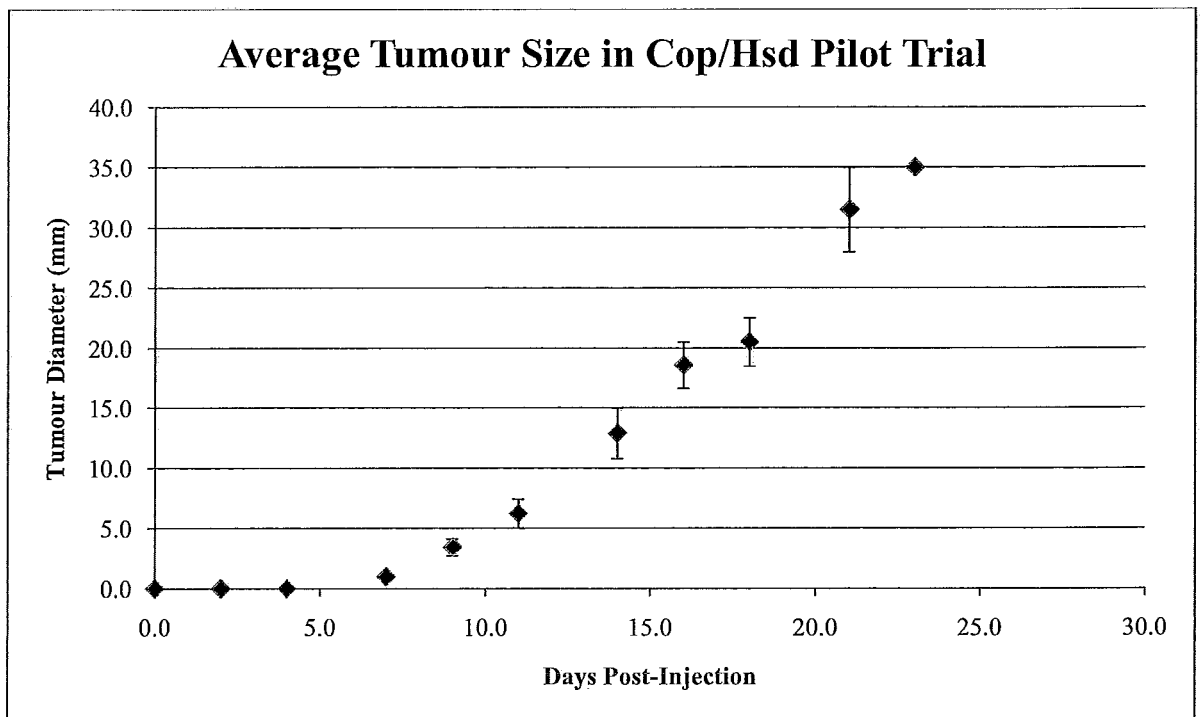


Figure 6 - Average MATLyLu Tumour Size in Cop/Hsd Rats with 35mm Diameter Endpoint

3.3 Design and Dosimetry of Rat Irradiator

Ionization chamber readings were collected at 0.5cm intervals in a matrix set up and arranged into a grid in Figure 7. Exposure given was 4 Roentgens at $d=26\text{cm}$. Recorded exposures ranged from 0 – 3.7 Roentgens. Exposures in Figure 7 are rounded to the nearest integer. “Point” (0,0) is at the corner of the irradiator opening, and markings around the sides are in cm relative to (0,0) (See design in section 2.3 for details).

	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4
-12.5	0	0	0	0	0	0	0	0	0	0	0	0	0
-12	0	0	0	0	0	0	0	0	0	0	0	0	0
-11	0	0	0	0	0	0	0	0	0	0	0	0	0
-10	0	0	0	0	0	0	0	0	0	0	0	0	0
-9	0	0	0	0	0	0	0	0	0	0	0	0	0
-8	0	0	0	0	0	0	0	0	0	0	0	0	0
-7	0	0	0	0	0	0	0	0	0	0	0	0	0
-6	0	0	0	0	0	0	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0	0	0	0	0	0	0
-4	0	0	0	0	0	0	0	0	0	0	0	0	0
-3	0	0	0	0	0	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0	0	0	0	0	0
-1	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 7. Dosimetry of Rat Irradiator. Open field indicated by box in bottom right. 4 Roentgen of exposure given and measured by 0.5cm x 0.5cm matrix (in open field, shielded portion was in 2cm x 2cm matrix). Values in figure are rounded to the nearest integer. Max exposure recorded was 3.7 R. Marking around side indicate coordinates (in cm), with the top-left corner of the open field designated (0,0).

3.4 Effects of Apocynin on Radiosensitization *In Vivo*

Groups which received apocynin via IP injection were excluded from statistical analysis.

Explanation for this can be found in section 4.4.

3.4.1 Survival Time

Survival time was measured in days from the day of MATLyLu tumour injection to endpoint.

Survival time was averaged for each group, including only rats who reached endpoint via tumour size $\geq 35\text{mm}$ or tumour ulceration. Rats which were euthanized for poor body condition, regrowth of excised left-tumour, leg paralysis, other non-tumour related reasons, or died naturally were censored from statistical analysis for survival time.

Figure 8 shows a Kaplan-Meier survival curve created for the treatment groups. Statistical significance was calculated using log-rank test, followed by pairwise multiple comparison by using Holm-Sidak method.

All groups which received radiation differed significantly from those that did not. The group which received radiation as well as a full 14-day course of apocynin, and the group which received radiation alone differed with an unadjusted p value of 0.0412, however this was not significant beyond the Holm-Sidak calculated critical level of 0.0127. Therefore there were no statistical differences among irradiated groups and among unirradiated groups.

A full list of p value comparisons between group survival curves can be found in Table 8.

3.4.2 Tumour Growth

Tumour growth was measured for each rat daily (Monday-Friday) until endpoint was reached. Growth is expressed as the average of each group over time in Figure 10. Significance was tested by chi-squared analysis from day 14 (first day of irradiation in irradiation groups) until endpoint was reached for both groups in each pairwise comparison. Rats which did not develop tumours were excluded from this analysis, as were outliers. No significant difference was found by adding apocynin alone ($p = 0.085$). Significant differences were found by adding radiation treatment in all comparisons ($p < 0.001$), except for the comparison of the group which received apocynin alone to the group which received radiation alone, between which no significant difference existed. Among irradiated groups, significant difference was not observed when treating with apocynin 5mM PO for 5 days or the full 14-day course of treatment. Table 9 shows the chart of p values for this comparison.

3.4.3 Doubling Time

Doubling time was measured by methods discussed in section 2.4.3. Rats which did not develop tumours, as well as outliers, were excluded from this analysis.

Doubling time in each irradiated group was significantly greater than for each unirradiated group ($p < 0.0001$). There were no statistically significant

differences among irradiated or unirradiated groups. Groups given radiation with apocynin for either five or fourteen days were then combined to compare all rats given radiation and apocynin (regardless of duration of apocynin treatment) to the group which received radiation and no apocynin. No statistically significant difference was found ($p = 0.0687$). P values are displayed in Table 10.

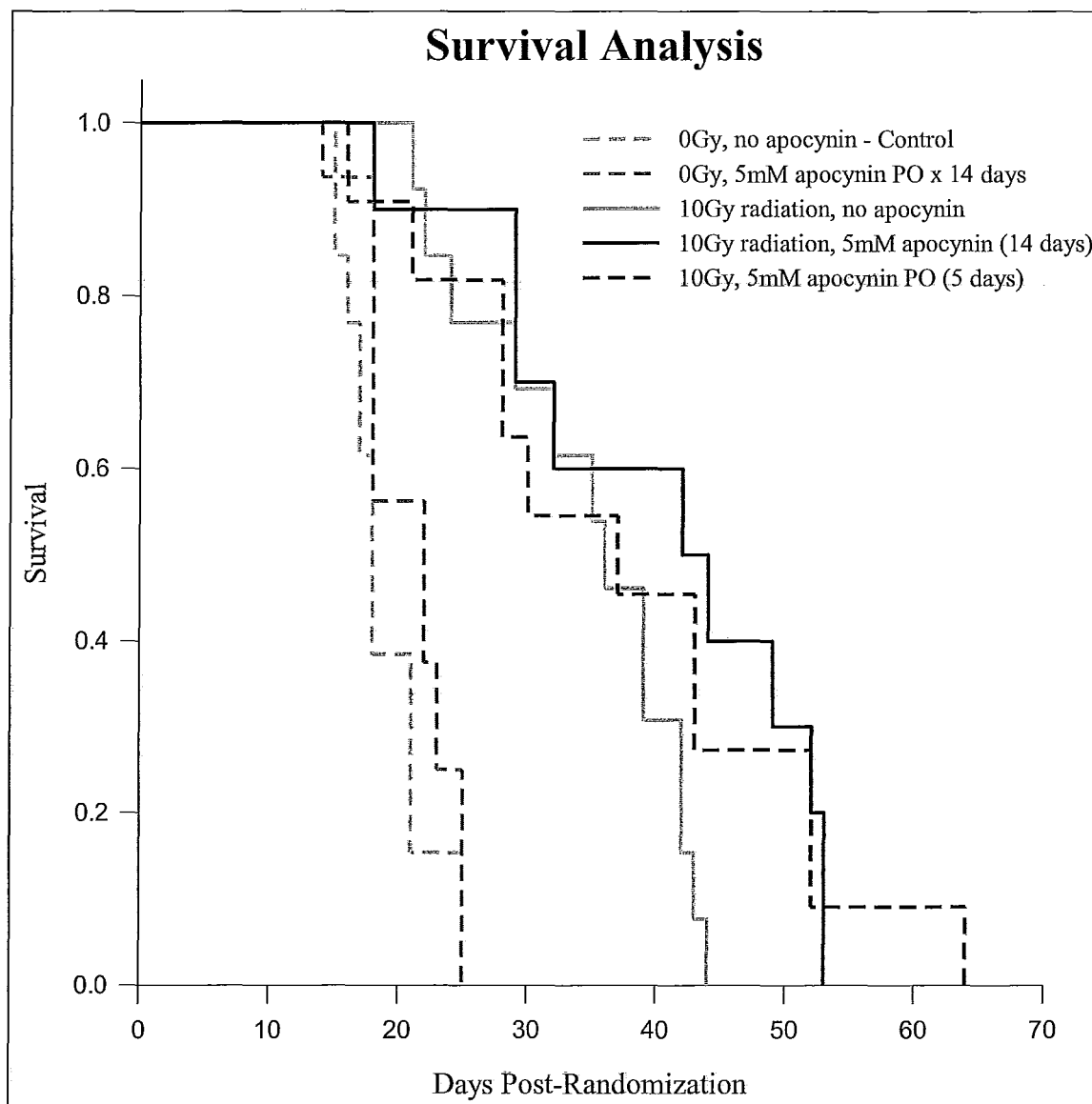


Figure 8 - Kaplan-Meier Survival Curve of Rats treated with different combinations of radiation and apocynin. The treatment groups were: 0Gy radiation and no apocynin control (light grey dashed line), 5mM apocynin orally for 14 days (black dashed line), 10Gy (2Gy x 5 days) irradiation alone (black solid line), 10Gy (2Gy x 5 days) irradiation with 5mM apocynin orally for 14 days (light grey solid line), and 10Gy (2Gy x 5 days) irradiation with 5mM apocynin orally for 5 days (grey solid line)

Table 8 - Statistical Significance in Kaplan-Meier Survival Curves in Treatment Groups

Multiple Comparisons:				
All Pairwise Multiple Comparison Procedures (Holm-Sidak method):				
Overall significance level = 0.05				
Comparisons	Statistic	Unadjusted P Value	Critical Level	Significant?
Con – IR	20.217	0.00000691	0.00512	Yes
Apo(14) – IR+Apo(14)	18.063	0.0000214	0.00568	Yes
Con – IR+Apo(14)	17.813	0.0000244	0.00639	Yes
Apo(14) - IR	16.869	0.0000401	0.00730	Yes
Con – IR+Apo(5)	15.576	0.0000793	0.00851	Yes
Apo(14) – IR+Apo(5)	14.419	0.000146	0.0102	Yes
IR – IR+Apo(14)	4.141	0.0418	0.0127	No
Con – Apo(14)	2.491	0.115	0.0170	No
IR vs. IR+Apo(5)	1.583	0.208	0.0253	No
IR+Apo(14) vs. IR+Apo(5)	0.111	0.739	0.0500	No

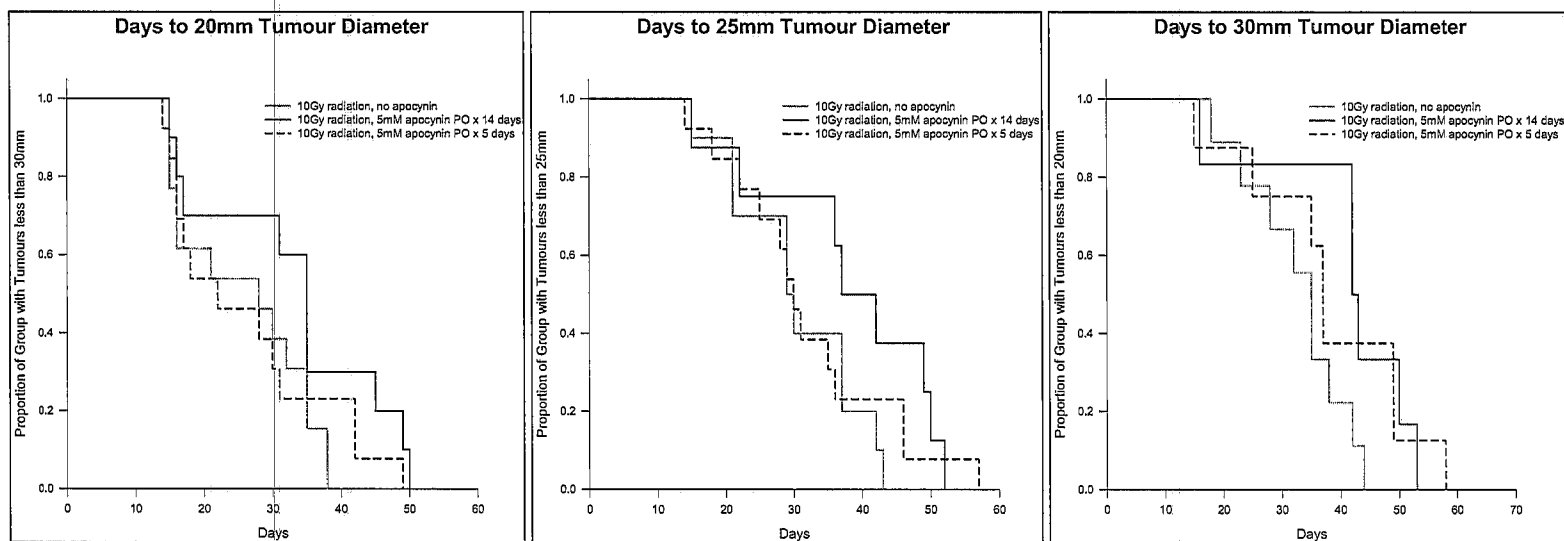


Figure 9 – Time in days taken for each radiation group to reach tumour diameters of 20mm, 25mm, and 30mm. No significant difference was found between 10Gy with no apocynin and 10Gy with 5mM apocynin PO x 14 days at 20mm ($p = 0.0792$), 25mm (0.0857), or 30mm (0.0514). All other comparisons were also non-significant ($p > 0.1$).

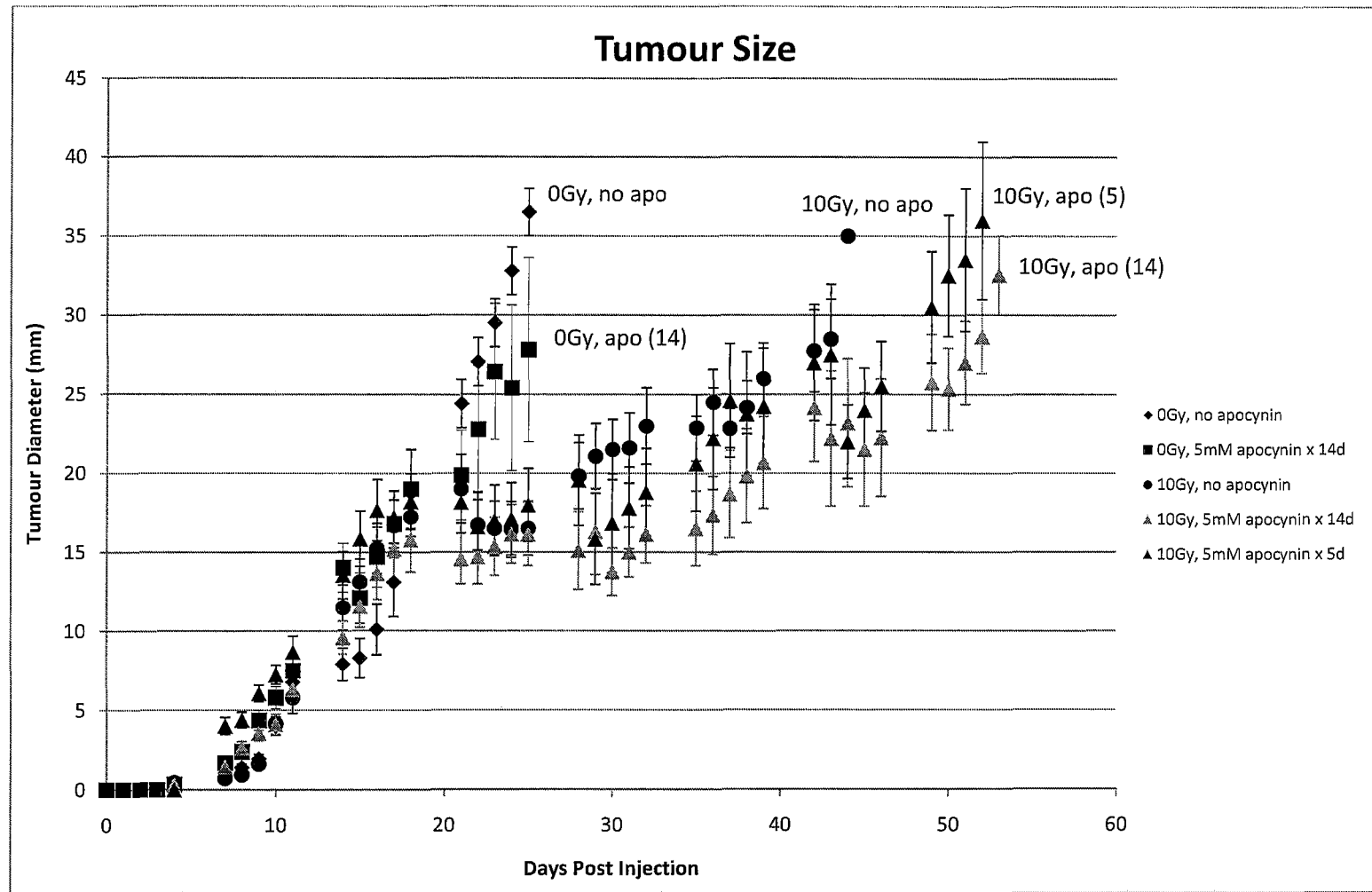


Figure 10 - MATLyLu Tumour Size in Cop/Hsd Rats

Table 9 - P Values for MATLyLu Tumour Growth Comparisons by Chi-Squared Test. The shaded cells represent comparisons that showed statistical significance.

	IR alone	Apo (14)	IR + Apo (14)	IR + Apo (5)
Control	1.61069E-05	0.085116433	1.61069E-05	0.000478676
IR alone	x	0.127364602	0.064087653	0.96198754
Apo (14)	x	x	1.26233E-08	1.95682E-08
IR + Apo (14)	x	x	x	0.795031694
IR + Apo (5)	x	x	x	x

Doubling Times for MATLyLu Tumour Growth in Cop/Hsd Rats

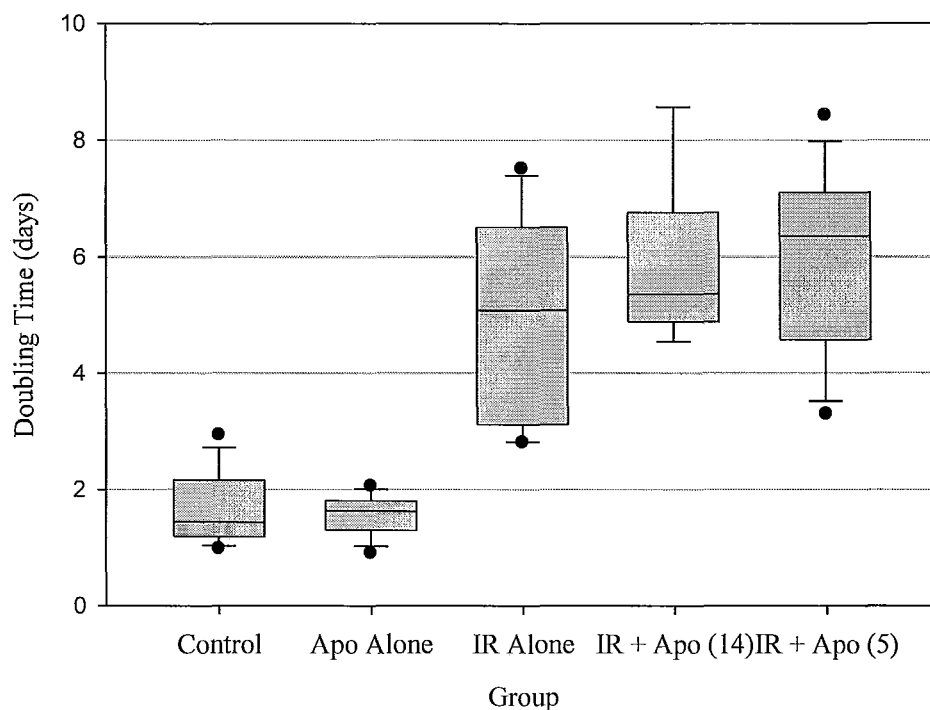


Figure 11 - Box Plot for MATLyLu Tumour Doubling Times; with rats with doubling times greater than two standard deviations from the mean ruled as outliers and removed from the analysis.

Table 10 - P Values for Doubling Time Comparisons by T-Test. The shaded cells represent comparisons that showed statistical significance.

	Apo Alone	IR Alone	IR + Apo(14)	IR + Apo(5)	IR + Apo(14) and IR + Apo(5)
Control	0.66002	1.01446E-06	2.45852E-09	1.36911E-09	7.33867E-12
Apo Alone	x	3.82847E-07	8.70194E-10	2.91357E-10	1.02514E-12
IR Alone	x	x	0.183451607	0.114799022	0.068718531
IR + Apo(14)	x	x	x	0.880766961	x
IR + Apo(5)	x	x	x	x	x

3.5 Effects of apocynin on ROS production in MATLyLu cells *In Vitro*

ROS production was measured in MATLyLu cells cultures *in vitro* with and without apocynin as described in section 2.6. NBT assay was run twice with complementary concentrations of apocynin; first with concentrations of 0 μ M, 200 μ M and 1000 μ M, then with 0 μ M, 100 μ M, 500 μ M, and 2000 μ M. Each concentration point is the average of 21 wells that were treated with the given concentration of apocynin (or DMSO for second control). Each case was normalized to the 0 μ M sample for the respective experiment. A significant reduction in total ROS production was found at 500 μ M ($p = 0.012$), 1000 μ M ($p = 0.0064$), and 2000 μ M ($p < 0.0001$), as shown in Figure 12. The relationship appears to be quite linear in this range, such as is shown in Figure 13 with a correlation of determination of 0.953.

3.6 Effects of apocynin on cell proliferation in MATLyLu cells *In Vitro*

Cell proliferation was measured in MATLyLu cells *in vitro* with and without apocynin as describes in section 2.7. The WST-1 assay was run twice with each concentration point averaged among 21 wells that were treated with the given concentration of apocynin (or DMSO for second control). Each case was normalized to the 0 μ M sample. A significant reduction in cell proliferation was found at 500 μ M ($p=0.0056$), 1000 μ M ($p<0.0001$) and 2000 μ M ($p<0.0001$), as shown in Figure 14. When ROS production was normalized to these findings on the effects of apocynin on MATLyLu proliferation, there was no significant reduction in ROS production per cell (Figure 15), and perhaps an increase at high doses.

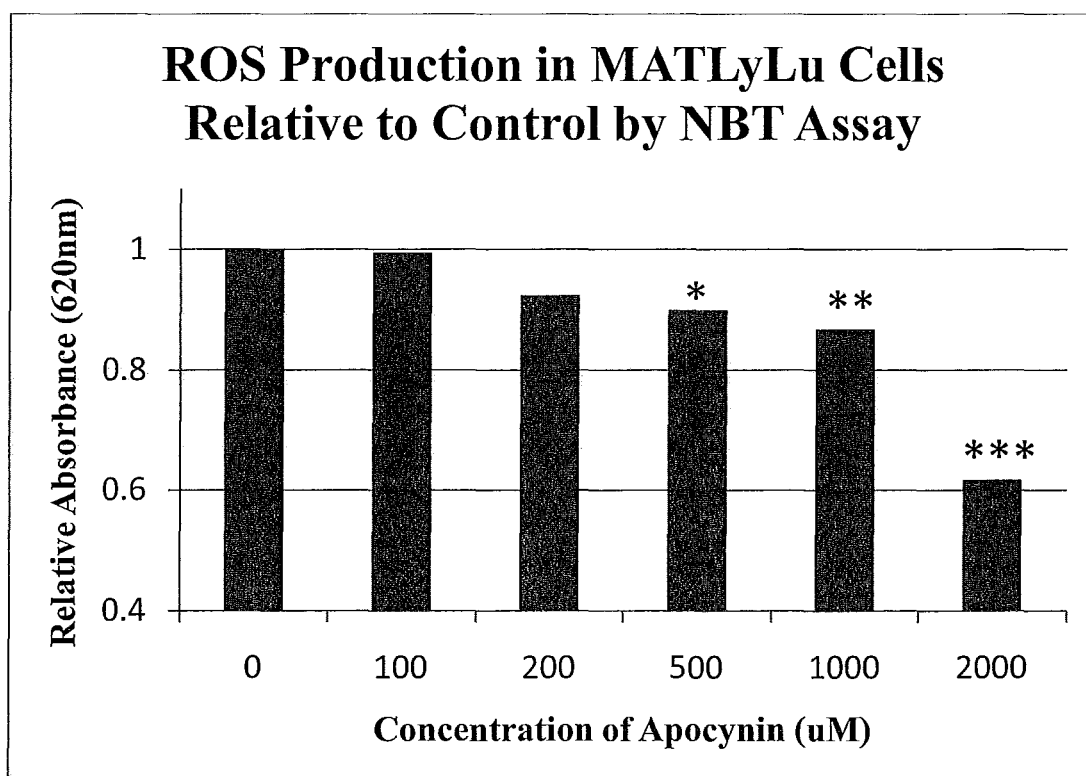


Figure 12 - Effects of apocynin on total ROS production in MATLyLu cell cultures. NBT forms yellow formazan crystals when incubated in ROS-rich environments, such that lower ROS concentrations will yield a paler medium, and a corresponding lower spectroscopy absorbance reading. *, **, and * represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.**

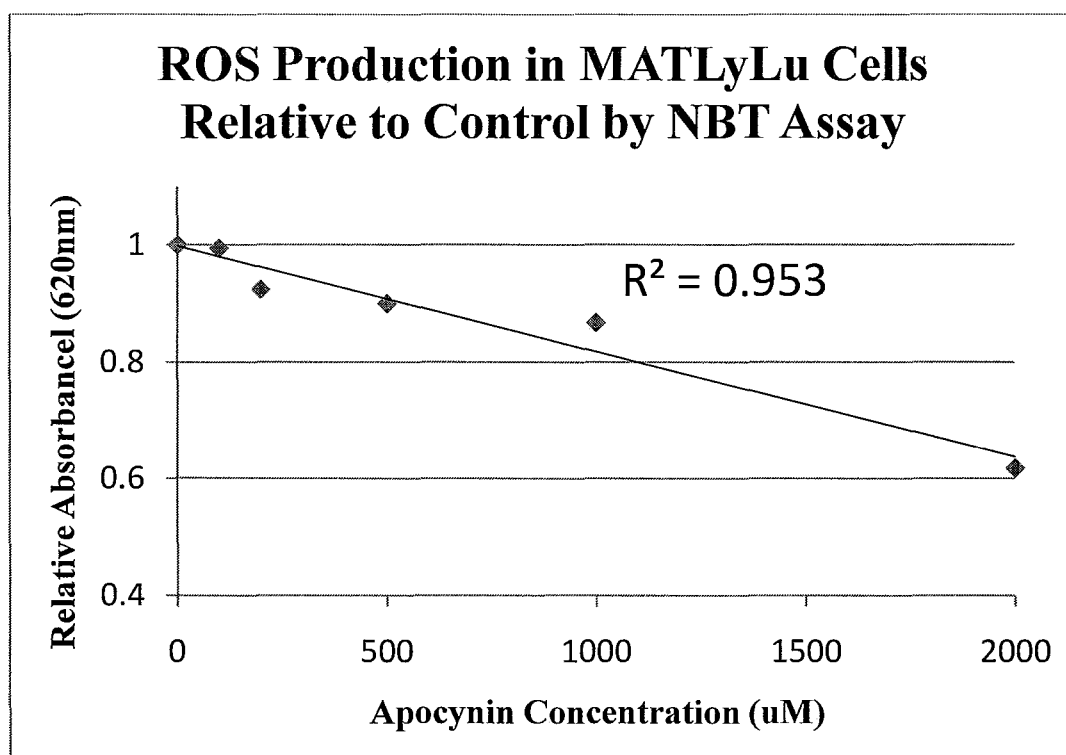


Figure 13 - Effects of apocynin on total ROS production in MATLyLu cell cultures

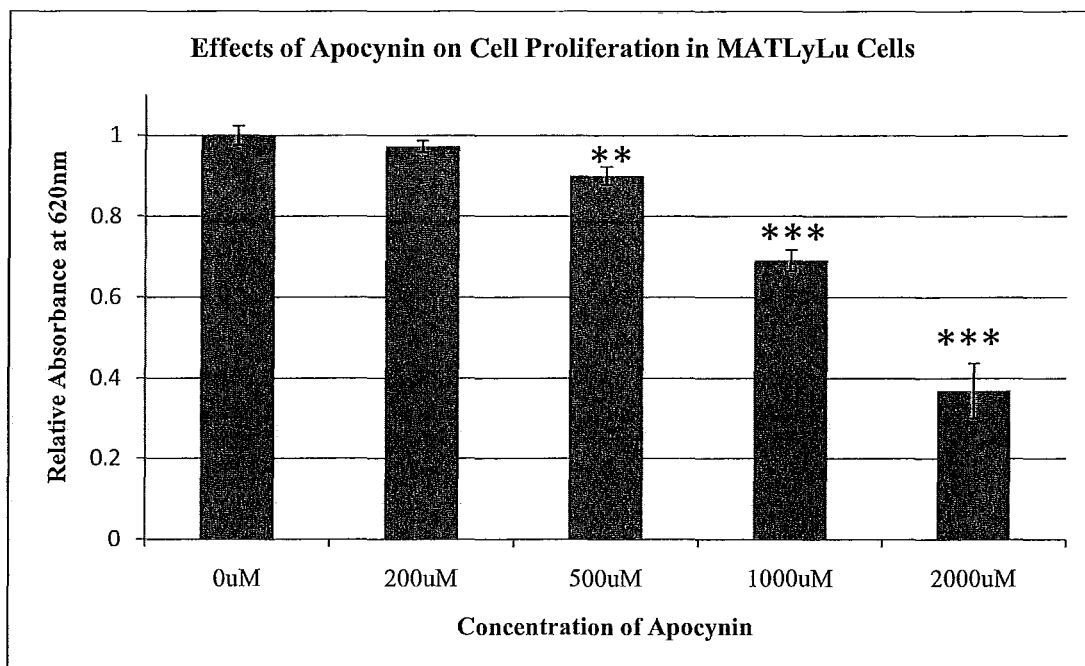


Figure 14 - Effects of Apocynin on Cell Proliferation in MATLyLu Cells. The WST-1 reagent forms a dark yellow solution in highly proliferative cultures, such that a paler solution indicates non-proliferation, likely attributable to cell death. *, **, and * represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.**

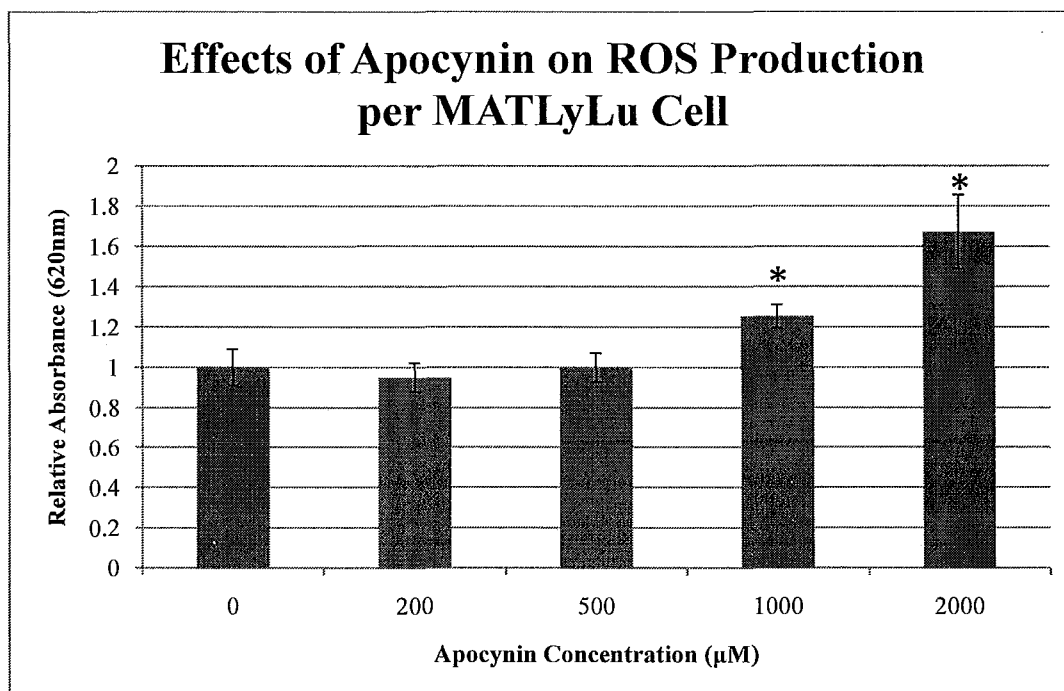


Figure 15 - Effects of Apocynin on ROS Production per MATLyLu Cell. Findings represent the effects of apocynin on relative ROS production in MATLyLu cells (Figure 12) divided by the relative rate of cell proliferation (Figure 14). No effects was found on ROS production per cell at lower concentrations, and it appears there is evidence of an increase in ROS production at higher concentrations, although these may be outside the range of accurate measurement by the WST-1 assay.

3.7 Effects of apocynin on MATLyLu Survival Post-Irradiation *In Vitro*

The effects of apocynin on the radiosensitivity of MATLyLu cells were determined by methods A, B, and C, all outlined in section 2.8. All cell counts (method A) were the result of a single flask, while all colony formations counted (Methods B and C) were the result of flasks averaged either in triplicate or quadruplicate. Development of irradiation dosing regimens followed an apocynin-free trial using method A, in which the surviving fraction of MATLyLu cells was determined following radiation doses of 0Gy, 2Gy, 4Gy, 6Gy, 8Gy, and 10Gy, by Method A (data not shown). It was found that the dose needed to kill approximately 50% of the colonies (LD_{50}) was approximately 3Gy. Later studies using 3Gy with Method B confirmed that 3Gy was a reasonably accurate estimate for LD_{50} . ($53.5\% \pm 2.6\%$).

Colony formation assays (Method B and C) were run on MATLyLu cells incubated for 24 hours with 0 μ M, 200 μ M or 1000 μ M apocynin, and irradiated on ice with 0, 1.5, 2, 3, or 10Gy. Results for apocynin-treated groups were relative to their respective 0Gy cohort and comparisons between groups were made by comparing the fraction of colonies formed at 1.5, 2, 3, or 10Gy, vs. 0Gy with the same apocynin dose. One study showed a significant survival decrease at 0Gy with 1000 μ M apocynin ($p = 0.0128$), and at 3Gy with 200 μ M apocynin ($p = 0.033$), however the values were quite erratic with very wide spreads. Another study showed a significant survival decrease at 2Gy with 1000 μ M apocynin ($p=0.0348$), however the cause of increased surviving fraction was not in a reduction of colonies formed at 2Gy, but in an increase in those formed at 0Gy (Figure 16).

Earlier attempts by Method A were able to demonstrate a significant reduction in cell survival at 2Gy with 200 μ M apocynin ($p = 0.0420$) when the cultures were allowed to grow for 80 hours post-irradiation, and a near-significant reduction when allowed to grow for 106 hours ($p = 0.0679$).

A summary of all pairwise survival comparisons can be found in Table 11.

Based on these findings, there is no conclusive evidence to show that apocynin at 200 μ M or 1000 μ M successfully sensitized MATLyLu cells *in vitro* to radiation.

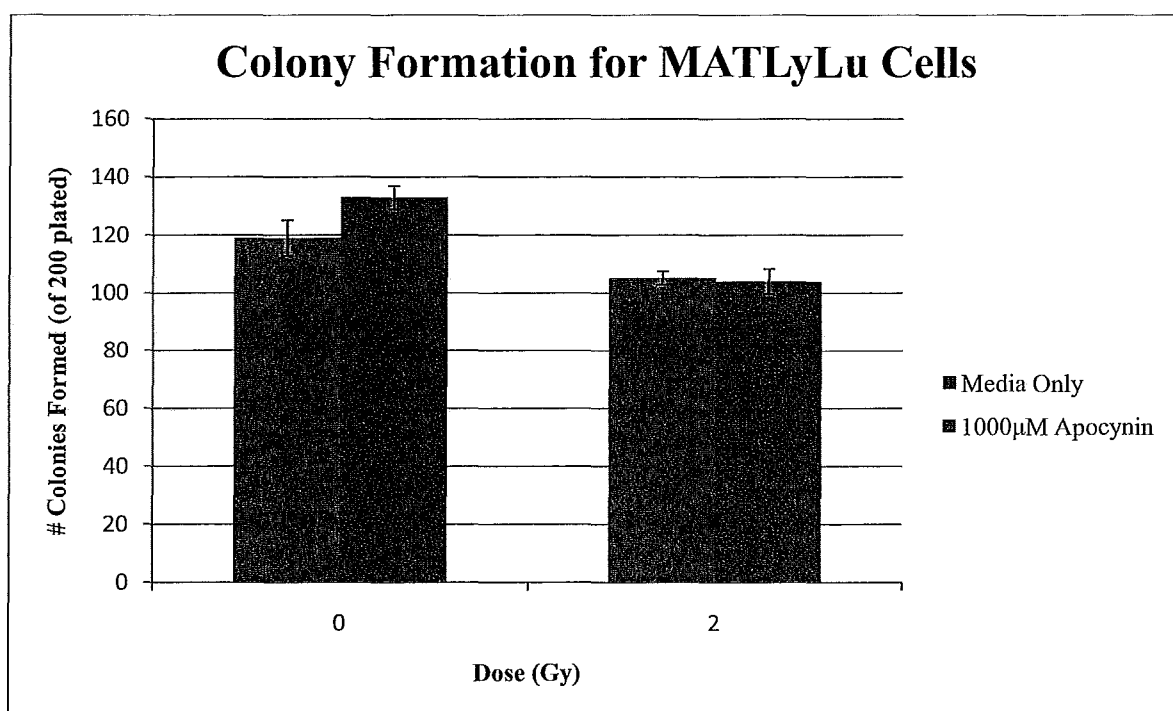


Figure 16 - Colony Formation Assay with MATLyLu Cells by Method C. Plating efficiency across all colony formation assays at 0Gy was 60-70% in each trial.

Table 11 - Summary of *In Vitro* MATLyLu Survival Assays. Shaded cells indicate tests which produced statistically significant differences. Notes in the right column explain briefly why statistically significant comparisons might not indicate radiosensitization by apocynin. Trials with dose ranges indicated (i.e. 0-8 or 0-10) involved survival curves with dose points spaced 2Gy apart, with statistics calculated by Chi-squared analysis. Trials with single dose points had statistics calculated by Student's two-sided t-test.

Gy	uM	Method	%Apo	%Med	p	Comments
0-8	200	A	n/a	n/a	0.922879	
10	200	B	0.45	n/a	0.462142	
2	200	A	0.882974	1.006578	0.042026	80hr incubation
2	200	A	0.803251	0.844317	0.067846	106hr incubation
0-10	200	A	n/a	n/a	0.999911	*Apocynin given for 72 hours pre-rads
0-10	200	A	n/a	n/a	1	*Apocynin given for 72 hours post-rads
0-10	200	A	n/a	n/a	0.999998	*Apocynin given for 72 hours pre- and post-rads
0	200	B	0.910828	1	0.186267	
3	200	B	0.535032	0.496815	0.192116	
0	200	B	1.296491	1	0.359872	
0	1000	B	1.896491	1	0.012811	Erratic values
1.5	200	B	1.445614	1.352632	0.96999	
1.5	1000	B	1.764912	1.352632	0.218951	
3	200	B	0.910526	1.563158	0.033298	Erratic values
3	1000	B	1.250877	1.563158	0.241966	
0	1000	C	1.117647	1	0.103692	
2	1000	C	0.781955	0.884454	0.034841	*SF not down, initial up - and all cells from same stock. Not a valid explanation.

Discussion

4.1 *In Vivo* Effects

When apocynin was administered to MATLyLu tumour-implanted Cop/Hsd rats *in vivo* prior to radiation treatment, a significant difference in survival was detected only when the apocynin was delivered at 5mM orally in their drinking water for a 14-day course of treatment (spanning 5 days before, 5 days during, and 4 days after radiation treatment) compared to the group which received apocynin at the same regimen but without radiotherapy. However, the difference was not significant beyond the critical level established by the Holm-Sidak method during pairwise comparison, which accounts for the probability that among a selection of random trends, one may differ from one another by chance. Nevertheless, a one-to-one comparison of the groups offers a fair comparison, and combined with the near-significant differences found between the groups at tumour diameters of 20mm, 25mm, and 30mm ($p = 0.0792$, $p = 0.0857$, $p = 0.0514$, respectively), it is unlikely that the difference in overall survival is attributable to chance. Meanwhile, a significant difference in survival was not detected between groups which received apocynin at the same oral 14-day course of treatment and which received no apocynin in the

absence of radiation. It appears, then, that the combination of apocynin and radiotherapy confers a greater survival advantage than either therapy on its own.

Other apocynin-radiation combined treatment regimens were unable to produce statistically significant differences in survival; however the group which received the shortened regimen of oral apocynin consistently demonstrated results in between the radiation control and the radiation with 14-day apocynin group, indicating a potential trend in radiation-apocynin combined effectiveness as apocynin administration is more closely coincident with radiotherapy in timing.

The group treated with apocynin at 5mM orally for only 5 days prior to radiation did appear to show marginal (but non-significant, $p = 0.208$) improvements in survival. In fact, while there was not a significant improvement in survival over the radiation only control group, there was also not a significant decline in survival compared to the 14-day treatment group, and the results are graphically visible in Figure 8 in between the two groups.

The observations here suggest that the addition of apocynin to groups treated with radiation improves the response to radiation, and perhaps in a dose- or timing-dependent fashion.

Two interpretations of this effect are appropriate.

First, that the addition of apocynin to groups treated with radiation is improving the sensitivity of the implanted MATLyLu tumours to radiation treatment. This interpretation is consistent with the results found *in vivo*, mostly in that rats which received apocynin alone did not demonstrate a statistically significant change in survival over controls, while those which received a combined apocynin-radiation treatment regimen did demonstrate significant improvements over the radiation control. The NADPH-oxidase inhibiting mechanism of action for apocynin is also

consistent with these findings, as there was no evidence found in the literature that apocynin might on its own effect the proliferation of neoplastic tissues, and that its known effect is to down-regulate basal ROS defence mechanisms which would have direct implications for the susceptibility of the tissues to damage by radiation.

The second interpretation is that the observed effect is not on account of changes in radiosensitivity, but that the improved survivability of rats which received a combined apocynin-radiation treatment is a result of additive effects of apocynin and radiation treatment. This is supported by our *in vitro* findings, which demonstrate a significant inhibition of the rate of growth of MATLyLu cells treated with apocynin alone, and that a ROS-inhibiting effect in MATLyLu cells is not achieved by administration of apocynin. Also, while no significant improvement in survival *in vivo* was achieved by apocynin treatment alone, it is possible that its lesser significance (0.115 versus 0.0418 in the combined apocynin(14-day)-radiotherapy group) is attributable to the increased rate at which those rats reached endpoint, and that the effects of apocynin were only beginning to take place. This interpretation could be further investigated by increasing the dose or length of time for which apocynin is administered without radiation to Cop/Hsd rats transplanted with MATLyLu tumours.

The findings that the group treated with the 5-day apocynin regimen also showed survival benefits, albeit marginal and non-significant, might also support either interpretation. This group had its apocynin administration stopped precisely when the first fraction of radiotherapy was delivered. So on one hand, it is feasible that the lessened improvement in survival can be explained by the rate of biological clearance of apocynin, such that each day's course of radiotherapy would be sensitized to a lesser degree than the previous day (with the exact rate depending on the rate of clearance), summing to a decreased net sensitization effect over the

course of radiation treatment. Conversely, it is also feasible that the five-day apocynin regimen simply results in the rats ingesting nine-days-worth less of apocynin, a direct effect of which would be lessened inhibition of tumour growth.

Some insight into these conflicting views may be achievable by administering apocynin only for the five days of radiotherapy, or only the five days after, with the appropriate control groups. If an improvement over the radiation control is seen only in the group which has coincident apocynin and radiation treatment, the effect might be attributed to sensitization, and if an equal effect is seen in both groups, then the additive mechanism becomes more likely.

Without such an experiment, however, speculation into the shortcomings of each interpretation suggests that the sensitization model is more probable.

Our findings which show that apocynin does not inhibit ROS production (and thus does not confer a radiosensitization effect) in MATLyLu cells *in vitro* are not necessarily applicable *in vivo*. As is discussed in greater detail in section 4.2, there is a significant body of literature which shows that the NADPH-oxidase inhibiting effects of apocynin requires synthesizing it into its dimer form, diapocynin, and that this is a process which is not ubiquitous across cell lines^{53,55,59,60,61}. Rather, it has been shown with a degree of consistency that apocynin inhibits NADPH oxidase only in cells which inherently possess peroxidises which are perhaps the responsible agents for the synthesis of the active form, diapocynin. Indeed, the artificial means for producing diapocynin require reactions involving peroxidises^{69,71}. Similarly, studies have suggested that the synthesis of apocynin into diapocynin occurs during ingestion and digestion^{64,65,66,67}. (This is also perhaps part of the reason why rats which received apocynin by injection did not demonstrate any survival benefits, which is also discussed shortly.) It should first be ascertained whether or

not MATLyLu cells do inherently possess peroxidises; but if they do not, it is a feasible explanation that their non-effect *in vitro* is a result of apocynin being inactive in its natural form, and that its effect on implanted MATLyLu tumours *in vivo* is owing to its conversion to diapocynin during ingestion. These considerations, however, only discount the otherwise contradictory findings between our *in vivo* and *in vitro* results. At this point, it would be necessary to assess the effects of diapocynin on MATLyLu cells *in vitro*, and further consideration on the *in vivo* effects of ingested apocynin (or diapocynin) alone are still required.

Therefore, while a sensitization effect may be more easily explained by the known mechanisms of apocynin, it is only possible to conclude based on our findings that the combination of apocynin and radiation treatment do improve survival overall radiation treatment alone, and the exact mechanism must be investigated further.

The group which received apocynin via 14 daily intraperitoneal injections (5mg/kg) did not show significantly different survival with or without concurrent radiation treatment. This lack of effect can be explained two different ways: First, the apocynin was not delivered to the intended concentration because its solubility in saline is unstable at the concentrations required for injection. Over 30% (0.151g of 0.5g apocynin) of the apocynin was recovered as a precipitate from the vial after the course was complete; apocynin dissolves well in high temperature water, but slowly reprecipitates out at room temperature when agitated. Second, as mentioned above, it may be possible that apocynin requires ingestion and digestion to be converted to its active dimer form. One study considering this possibility showed that diapocynin is found in the plasma of rats which ingested apocynin, and not in those which received apocynin by injection.⁷⁰ Either or both of these explanations adequately describe why an effect was not observed in the group which received apocynin by intraperitoneal injections.

The potential radiosensitizing effect of apocynin was hypothesized to be a result of NADPH oxidase inhibition, similar to the case of androgen deprivation. Preliminary Western Blot experiments for the quantification of p22-phox protein levels showed a significant decrease in p22-phox levels in MATLyLu tumour samples extracted from rats treated with 5mM apocynin PO for two days versus rats who had no apocynin. The results were significant when comparing quantified p22-phox bands from Western Blot gel electrophoresis at both peak and average levels between the mentioned groups. Apocynin was previously known only to prevent p22-phox from properly binding to NADPH oxidases, however one study has also shown that apocynin affects p22-phox mRNA expression⁷², so it may be possible that both mechanisms of action occur. However this finding requires further investigation; and while the reduction of p22-phox protein levels on MATLyLu cells would account for the increased radiosensitivity *in vivo*, it is not possible at the present to conclusively state that this mechanism of action is responsible for the radiosensitizing effects seen. These results are displayed in appendix A.2.

If it is true that the survival effects of apocynin are ascribable to radiosensitization, it is then important to determine whether this effect is unique to the prostate or to malignant cells in general. It is a fundamental tenet of radiosensitizers that their effect be exclusive or at least more pronounced in the target tissue (tumour) compared to normal tissues that will also be in the radiotherapy field. Otherwise, it becomes as effective a technique to merely increase the radiation dose. It has been shown that another potent NADPH oxidase inhibitor, parthenolide, inhibits NADPH oxidase selectively in PC3 prostate cancer cells, and not in healthy prostate tissue or other healthy tissues in the area⁷³. A simple analysis was performed to determine if apocynin was affecting the radiosensitivity of the skin as much as the implanted MATLyLu tumours by comparing the number of ulceration events between the group which received radiation alone and

the groups which received apocynin with radiation. No significant difference was found in the incidence of ulcerations or in the time of their occurrence post-injection. However, sample sizes for this analysis were small (5, 6, and 5 rats in three different groups), so further experimentation should be performed to conclusively show that apocynin does not aid in the development of tumour ulcerations post-radiotherapy.

4.2 *In Vitro* Effects

When real-time polymerase chain reaction (RT-PCR) was performed on MATLyLu cells, NOX1, NOX2, NOX3, and NOX4 NADPH oxidase isoform activity was positively identified by replication of base sequences outlined in section 2.5. **This shows that the MATLyLu cell line transcribes the gene for p22-phox. Although we have not examined the MATLyLu cells for the presence of the p22-phox protein, the observed transcription of the p22-phox gene suggests the p22-phox protein is present in these cells and the MATLyLu cell line is a feasible cell line to use for experiments involving NADPH oxidase inhibition by apocynin or other compounds which prevent the proper function of the p22-phox subunit.** This experiment was performed by Zhefeng Hou at Dr. Jehonathan Pinthus's lab at the Juravinski Cancer Centre (Hamilton, ON), and the results are shown in Appendix A.1.

When apocynin was administered to MATLyLu cells first, followed by irradiation 4, 24, or 72 hours later, most combination treatments produced the same surviving fraction as radiation alone, regardless of the radiation dose or drug concentration used. Instances in which a significant

decrease was achieved were not reproducible, or did not correspond with any potential trend between timing of administration or dosage with radiosensitization effects, which were otherwise zero. Three different methods were utilized to carry out these experiments as outlined in section 2.8.

When apocynin was administered to MATLyLu cells by Method A, no significant difference in cell culture concentration was found following 0Gy, 2Gy, 4Gy, 6Gy, 8Gy, or 10Gy when apocynin was added at 200 μ M 72 hours before irradiation, immediately following radiation for 72 hours, or both. The finding that adding apocynin after irradiation produced no change indicates that any effect that apocynin might have is not due to proliferation effects or changes to DNA repair that might render surviving irradiated cells more or less viable. The finding that adding apocynin prior to radiation produced no change suggested that it is not capable of reducing NADPH oxidase activity in such a manner that would affect radiosensitivity as seen in previous studies with androgen deprivation³⁶. However, it was suggested by peers that this design would not control for proliferation effects, and that a standard survival colony assay should be employed as well.

When apocynin was administered to MATLyLu cells by Method B (standard survival colony assay) at 200 μ M or 1000 μ M either 4 hours or 24 hours prior to irradiation, no reproducible significant differences were achieved. This again suggested that NADPH oxidase inhibition in MATLyLu cells was not being achieved, likely due to the non-conversion of apocynin into its active dimer form, diapocynin. Method C was suggested, which would reverse the order of seeding cells with irradiation, such that cells were seeded 24 hours before irradiation as opposed to after. Again, no significant differences were observed in the surviving fraction between combined apocynin-irradiation groups and radiation alone.

One noteworthy observation in using Method C is that the cells appeared more resistant to radiation and formed colonies more readily than in Method B. This can be explained by the probability of eradicating colonies of different size. In Method B, each potential colony is grown from a single pre-irradiated cell, which will either proliferate into a colony or not. In Method C, individual cells are allowed to grow 24 hours before irradiation. MATLyLu cells have a doubling time of approximately 11 hours, so taking into account time to settle and any post-seeding delays in proliferation, each potential colony by Method C could be anywhere from 1 to 4 cells in size. Colonies of 2-4 cells would be statistically much less likely to be completely killed than the colonies of 1 cell used in Method B.

Based on all *in vitro* survival assays, it appeared that apocynin was not successfully inhibiting NADPH oxidase activity in MATLyLu cells. This finding was confirmed by use of the NBT and WST-1 assays, which showed that while apocynin did have inhibitive effects on total ROS production in MATLyLu cells *in vitro*, this was not true on a per-cell basis. Apocynin did not appear to affect cell proliferation in any of the surviving fraction experiments, but in the smaller cultures used in the WST-1 assay, there was a strong correlation between apocynin concentration and reduced cell proliferation. When analyzing both results together, it was evident that there were no significant differences identified for ROS production as measured by the NBT assay per viable cell in cells cultured with apocynin at 0 μ M, 100 μ M, 200 μ M, 500 μ M or 1000 μ M. At 2000 μ M there was an increase in ROS production per cell, although both the NBT and WST-1 assays are less linear as the concentration of cells approaches zero, so this finding would require further investigation.

Our findings then show that there is not an inhibitory effect on the production of ROS in MATLyLu cells by apocynin *in vitro*. This is in contrast with previous studies using the 22rv1

human prostate cancer cell line, where a significant decrease in ROS production as well as a significant degree of radiosensitization were both achieved at a dose of $200\mu\text{M}$ ³⁶. Our experiments with apocynin and MATLyLu cells made use of identical methods as in the aforementioned 22rv1 study, except for the energy of radiation used. The 22rv1 cells were irradiated using a 1.25MeV cobalt-60 source, while the MATLyLu cells were irradiated with a 667keV cesium-137 source. The difference in these energies is unlikely to account for the difference in radiosensitization effect, which is attributed to inhibition of ROS which was not demonstrated in the MATLyLu cells anyways. Therefore, a different consideration of what might explain this difference is required.

As previously mentioned, other investigators have found that apocynin is not by itself an inhibitor of NADPH oxidase, but rather its dimer form diapocynin, as well as potentially other derivatives, are the responsible agent^{53, 58,59,60}. Many studies have shown that apocynin does inhibit ROS production, likely via NADPH oxidase inhibition, however mostly in vascular cells such as lymphocytes^{53,58,60}. It has been shown that many vascular cells, and other cells in which apocynin is effective for this intended use, possess peroxidases such as myeloperoxidase (MPO) that are required for the synthesis of diapocynin from apocynin⁵⁵.

It is possible, then, that the explanation for the difference in radiosensitizing effect between 22rv1 cells and MATLyLu cells may lie in their inherent differing capabilities to convert apocynin to its active dimer form. If this conversion is indeed a product of peroxidase-mediated interactions, there are two methods by which the hypothesis that diapocynin synthesis is required to achieve the desired effect can be further studied with respect to these cell lines.

First, it is possible to artificially synthesize diapocynin from apocynin, and then use diapocynin directly instead of relying on its synthesis *in vitro*. The process makes use of various peroxidises, lending credibility to the theory that peroxidises are the necessary agent for synthesis *in vitro*. Methods were carried out to attempt the synthesis of diapocynin by previously described methods (section 2.10) multiple times, however the resulting substance was found to be highly cytotoxic when added to MATLyLu cells in culture, and thus not appropriate for conducting future experiments. It is likely that our attempts at diapocynin synthesis were not successful, as the final product is described in the literature as white⁶⁹, whereas our final product was rusty in colour, likely owing to the use of iron(II) sulphate heptahydrate which appeared to persist in the compound despite our efforts. For future studies on the necessity of diapocynin to achieve the desired effect in MATLyLu cells, it would be important to acquire synthetically prepared diapocynin from a reliable source and assess its comparative capacity to inhibit NADPH oxidase in peroxidase-deficient cells.

Secondly, it would be valuable to assess the basal level of peroxidises in MATLyLu cells and compare it with that of 22rv1 cells and other cells in which apocynin-mediated NADPH oxidase inhibition has been successful. This may account for the difference in response between these two prostate cancer cell lines, as well as potentially give way to a predictive method for identifying cell lines and tissues in which apocynin would be synthesized into diapocynin, and thus would confer its NADPH oxidase inhibitory effects in its natural form.

Conclusions

The combined treatment of apocynin with radiation treatment improves the survival of Cop/Hsd rats implanted with MATLyLu tumours. This effect is statistically significant only when rats are administered 5mM apocynin drinking water for five days before radiation and five days during radiation (10Gy/5fx), and continued for four days after, but not when the apocynin solution was given only for five days before radiation.

Apocynin does not effectively inhibit NADPH oxidase activity in MATLyLu cells in vitro, possibly because they lack the endogenous peroxides required to synthesize apocynin into its active dimer. As a result, apocynin added to MATLyLu cell cultures does not result in an increased in sensitivity to lethality by radiation.

Appendix A

A.1 Measurement of NADPH oxidase function in MATLyLu cells *In Vitro*

Real-time polymerase chain reaction was run on MATLyLu cells grown in culture to confirm the gene expression of ROS-generating NADPH oxidases NOX1, NOX2, NOX3, and NOX4, using actin as a control. Each protein was positively identified on film as shown in Figure 17.

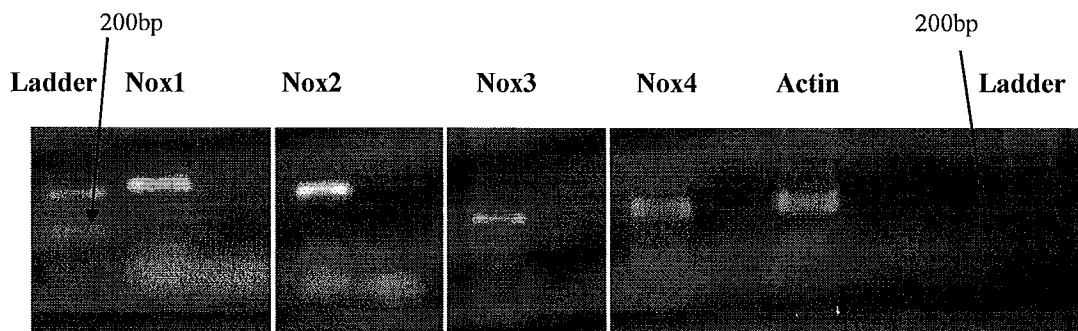


Figure 17 - Reverse Transcriptase Polymerase Chain Reaction of MATLyLu cells to confirm of NOX1, NOX2, NOX3, and NOX4 gene expression

A.2 Effects of apocynin on NADPH oxidase expression in MATLyLu Tumours in Cop/Hsd Rats *Ex Vivo*

Western Blot to detect the level of p22-phox proteins was run on tumour samples from rats with no apocynin in the control group and rats with exposure to apocynin in their drinking water for 3 days. Samples were run in triplicate at two different protein concentration levels. Bands were quantified and compared between the groups. A significant reduction was found in the peak ($p = 0.0233$) and average ($p = 0.0370$) band analysis between these two groups.

Control-12 Control-13 Control-14 Apo-9 Apo-10 Apo-11



Figure 18 - Western Blot Film for the Detection of p22-phox NADPH Oxidase Subunit

Table 12 - Band Quantification for p22-phox Western Blot Analysis. Shading in the cells showing P values for the comparisons indicated statistical significance.

RAT ID	p22avg	p22peak
CA12	958.284	1128.063
CA13	1070.058	1389.563
CA14	1870.841	1899.25
CC9	736.818	958.563
CC10	568.83	719.25
CC11	414.92	492.063
CC AVG	573.5227	723.292
CC_STDEV	161.0003	233.2763
CA AVG	1299.728	1472.292
CA_STDEV	497.7461	392.1931
P Value	0.037002	0.023364

Appendix B

Protocol for Biopsy Procedure

1. Anaesthetize rat at 3-4% isoflurane at 2L/min oxygen in induction chamber, and then maintain on same settings on surgical table.
2. Shave fur from incision sites on L leg and throat. See Figure 19.
3. Disinfect incision sites with iodine detergent and solution. See Figure 20.
4. Deliver 0.2mL buprenorphine (diluted 1:10 in saline) subcutaneously for 12hr pain control. See Figure 21.
5. Make incision around tumour using sterile surgical tools. See Figure 22.
6. Completely remove tumour as in Figure 23.
7. Split the tumour in two, and store half in formalin and half snap-frozen in liquid nitrogen. See Figure 24. Also collect a sample of shaved skin and store in each medium.

8. Suture leg incision using continuous under-skin method to reduce incidence of re-opening. Apply Vetbond skin glue after incision is fully closed. See Figure 25.
9. Make incision in throat approx. 1cm below notch in jaw as in Figure 26.
10. Remove left submandibular gland, ensuring to clamp its base before excising it to close prevent bleeding. See Figure 27.
11. Secure neck incision using sterile staples as in Figure 28.
12. Repeat steps 1-11 for all rats. Estimated time for each is 30 minutes

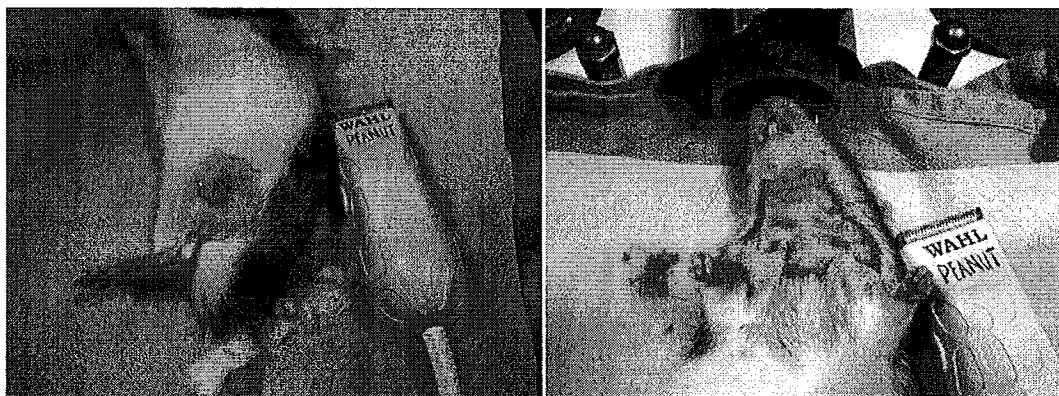


Figure 19 - Shave incision sites before beginning



Figure 20 - Disinfect area with iodine detergent and solution



Figure 21 - Disinfect incision site with iodine detergent and solution before making incision



Figure 22 - Make incision using sterile tools. Expose tumour.



Figure 23 - Remove tumour completely

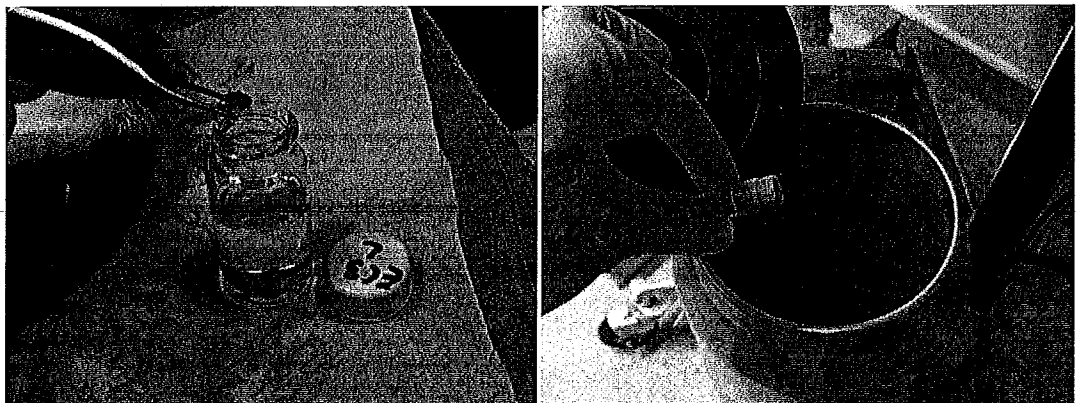


Figure 24 - Store half of tumour in formalin, half in liquid nitrogen. Collect a sample of shaved skin and do the same.



Figure 25 - Close incision using continuous under-skin sutures, and secure with Vetbond



Figure 26 - Make incision in throat 1cm below notch in jaw



Figure 27 - Exposure left submandibular gland, clamp its base, and excise it. Store in formalin and liquid nitrogen.



Figure 28 - Close throat incision using sterile staples

Rat Radiation Therapy Protocol

1. Transport rats in cages from CAF to GSB B105 Taylor Radiobiology Lab. Water bottles must be inverted, so they do not drip during transport, and cages must be covered. See Figure 29 for covered cages.
2. Arrange space in Taylor Source to have cages in systematic order, computer/books on desk space for recording data, closed circuit television to monitor rats during RT, and recovery cage with heating pad. See Figure 30.
3. Place irradiator platform beneath Taylor Source on alignment marks, and place irradiator on platform also on alignment marks, with shielding pieces accessibly nearby. The distance to the floor of the irradiator is 27cm. See Figure 31.
4. Open oxygen tank and open regulator to 1L/min. Fill halothane chamber with isoflurane and open to 4%.
5. Place rat in induction chamber. See Figure 32.
6. Once rat is anaesthetized, open airflow to nose cone/irradiator and place rat in irradiator as in Figure 33.
7. Place shielding over rat in irradiator as in Figure 34. Ensure ‘teeth’ of pieces overlap to minimize leakage as per Figure 35.

8. Calibrate dose for irradiation time. For 200cGy, use the following formula:
$$T = 420e^{((\ln 2/30)(x))}$$
where T is the time, in seconds, and x is the time in years after Nov 2009. E.g. For March 2010, $x=0.3333$, and $T=423s$.
9. Activate last-man button and sound pre-irradiation alarm as per Taylor Source SOP, and once the alarm has stopped, begin the irradiation. See Figure 36.
10. Place the irradiated rat in recovery cage. Repeat steps 5-9 for each rat.
11. Clean and secure Taylor Source room as per Taylor Source SOP, and return rats to CAF.



Figure 29 - Rats covered and transported from CAF to GSB B105 Taylor Radiobiology Lab

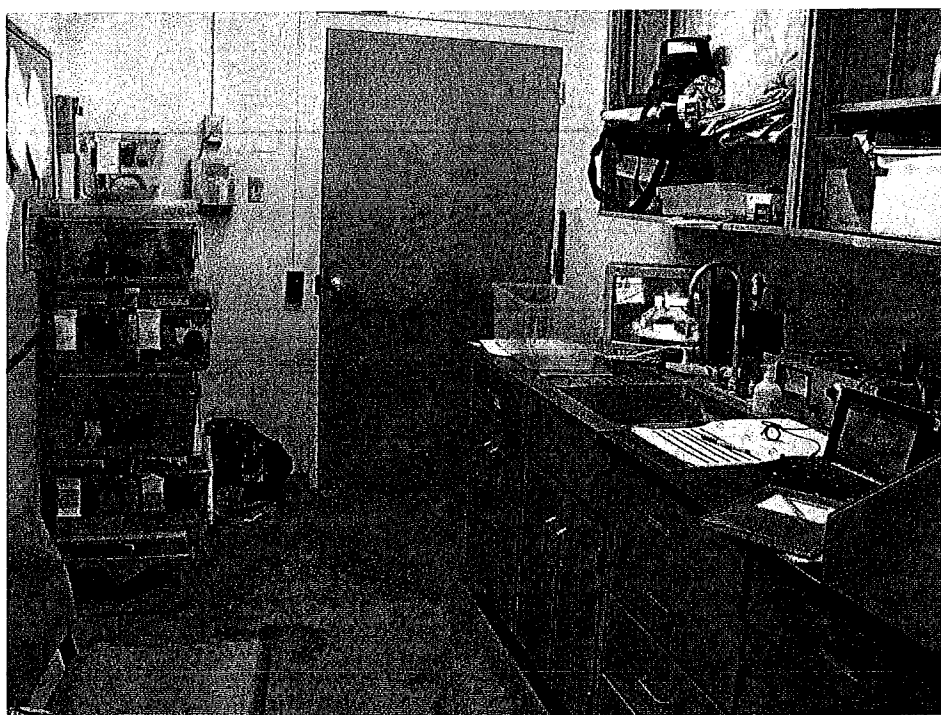


Figure 30 - Set up in GSB B105 Control Room

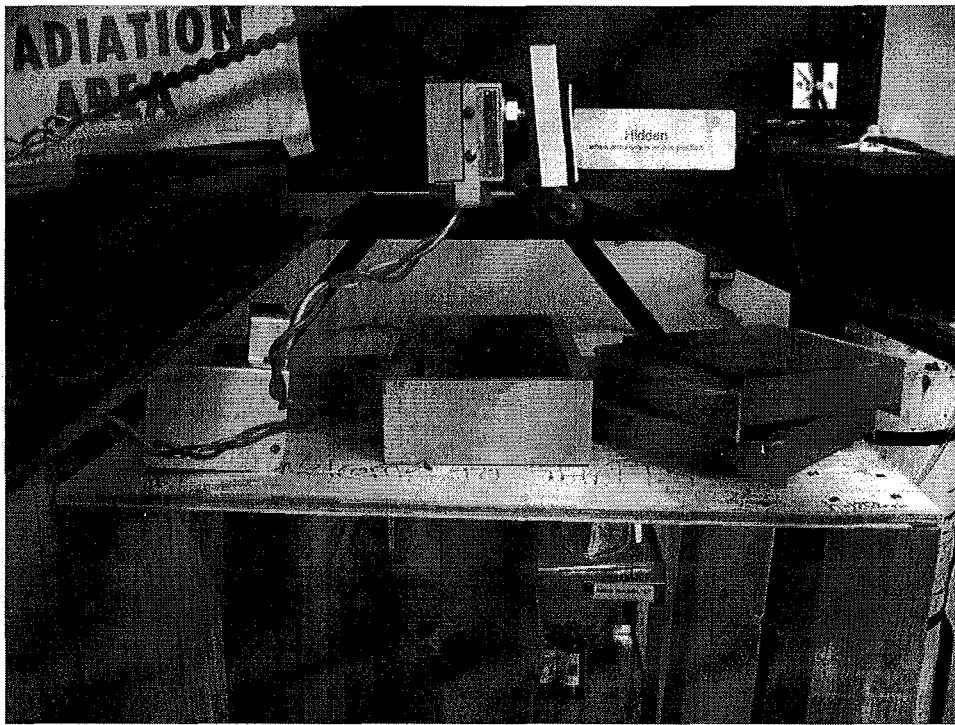


Figure 31 - Irradiator and shielding pieces on platform at $d=27\text{cm}$ from source

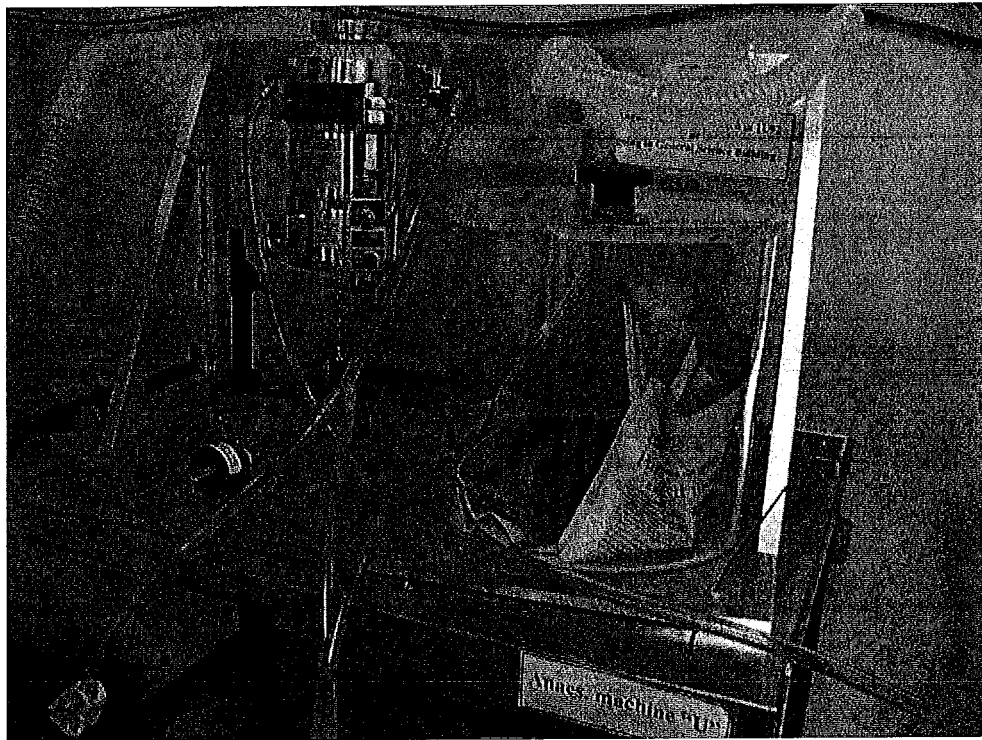


Figure 32 - Induction chamber with vaporizer set to 4% isoflurane at 1L/min oxygen



Figure 33 - Airflow diverted to nose-cone. Beam-eye-view of rat in irradiator without shielding

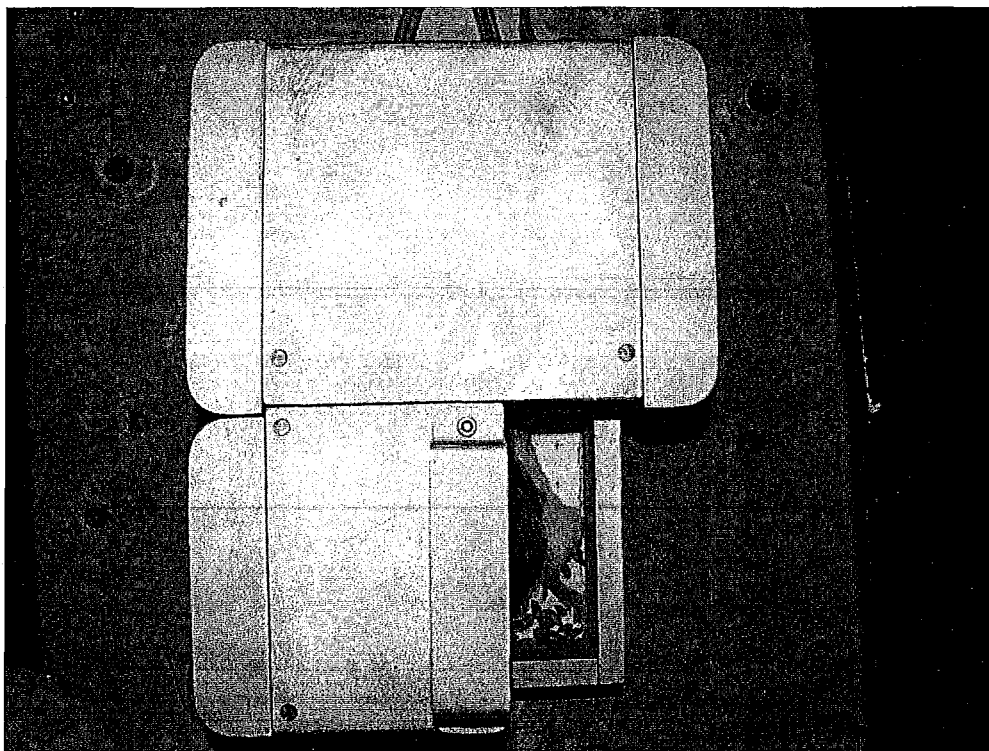


Figure 34 - Shielding placed on irradiator to expose only affected limb. Special attention made to fully shield spine and tail.



Figure 35 - Ensure 'teeth' of shielding blocks overlap to minimize leakage through shielding

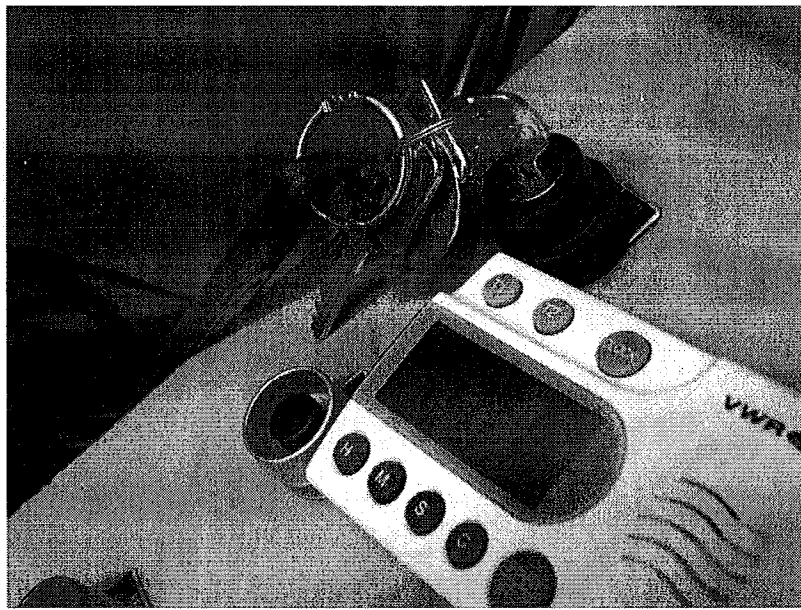


Figure 36 - Time set as per calculation, key in ready position

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