IN VITRO SENSITIVITY OF MURINE FIBROSARCOMA CELLS TO PHOTODYNAMIC THERAPY, ULTRAVIOLET LIGHT AND GAMMA-RAYS

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By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

Master of Science (1996)
(Biology)

McMaster University
Hamilton, ON

Title: In Vitro Sensitivity of Murine Fibrosarcoma Cells to Photodynamic Therapy, Ultraviolet Light, and Gamma-rays

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Number of Pages: i, 158

ABSTRACT

Photodynamic therapy (PDT) is a new form of cancer treatment that uses the localized delivery of light and a photosensitizing drug, which is selectively retained in tumor tissue, to cause photochemically induced cell death. Although PDT mediated by the sensitizer Photofrin (Ph-PDT) is currently in Phase III trials for a number of human cancers, the exact mechanism(s) involved in PDT induced cytotoxicity is not fully understood. Also, Photofrin has a number of drawbacks including extended cutaneous photosensitization and low absorption in the red region of the spectrum. This has lead to the search for improved sensitizers. In vitro, tumor cells resistant to PDT have been developed from PDT sensitive cell lines to examine the mechanism(s) of PDT action.

In this work, the sensitivity of RIF-1 murine fibrosarcoma cells and RIF-1 derived Ph-PDT resistant RIF-8A cells was examined following several damaging agents including PDT mediated by the novel Ruthenium phthalocyanine photosensitizer JM2929 (JM2929-PDT), UV, gamma-radiation, and hyperthermia. Gamma-radiation sensitivity of two other RIF-1 derived Ph-PDT resistant variants, CPR-C1 and RIF-P16CL8, was also examined. RIF-8A cells showed cross resistance to UV but increased sensitivity to gamma-rays compared to RIF-1 cells. RIF-1 and RIF-8A cells showed similar sensitivity to JM2929-PDT and hyperthermia. It is possible that Ph-PDT induces a "UV-like" component of damage and/or there is some overlap in the pathways for the repair of UV and Ph-PDT induced damage, but not JM2929-PDT, hyperthermia, and ionizing radiation damage in RIF-1 and RIF-8A cells. A cross resistance to gamma-rays

was observed for CPR-C1 but not RIF-P16CL8 cells. Since Ph-PDT resistant CPR-C1 cells, but not RIF-8A cells or RIF-P16CL8 cells, show a cross resistance to gamma radiation, these results suggest that the cellular changes required for RIF-8A, RIF-P16CL8, and CPR-C1 cells to become resistant to Ph-PDT are different. Survival of RIF-1 and RIF-8A cells following gamma-rays in the presence of either Photofrin or JM2929 was also examined. Results suggest sensitization of RIF-1 cells, but not RIF-8A cells, to gamma-radiation in the presence of Photofrin. Gamma-radiation in the presence of JM2929 had no sensitizing effects on the survival of RIF-1 and RIF-8A cells.

DNA repair of a UV-damaged reporter gene was also examined in untreated as well as Ph-PDT, JM2929-PDT, UV, cisplatin, and hyperthermia pretreated RIF-1 and RIF-8A cells. Results suggest an increased repair of UV damaged DNA in untreated RIF-1 cells compared to untreated RIF-8A cells. Ph-PDT, JM2929-PDT, and UV pretreatments resulted in an increased reactivation of a UV damaged reporter gene in RIF-1 cells compared to RIF-8A cells. Enhanced reactivation of a UV damaged reporter gene was not observed in either RIF-1 or RIF-8A cells following cisplatin or hyperthermia pretreatment. Enhanced expression of an undamaged reporter gene was greater in RIF-8A cells compared to RIF-1 cells following Ph-PDT pretreatment, but similar to RIF-1 cells following pretreatment with all other agents. These results suggest that the relation between survival, DNA repair of an actively transcribed gene, and transcriptional enhancement of an actively trancribed gene, varies in RIF-1 and RIF-8A cells depending on the damaging agent used. However, decreased reactivation of a UV

damaged reporter gene in RIF-8A cells may be related to Ph-PDT and UV resistance seen in RIF-8A cells.

PREFACE

This thesis is presented in 5 chapters. In the first chapter, an introduction to and review of general concepts in Photodynamic Therapy are discussed. The second chapter presents data obtained from initial experiments used to determine the appropriate technique for experiments described in chapters three and four. Chapter three is the first section of the study written in preparation to be submitted for publication. In this chapter, the sensitivity of murine fibrosarcoma cells following JM2929 mediated PDT, UV, gamma rays, and photosensitizer mediated gamma-ray sensitization was examined. The fourth chapter is the second section of the study also written in preparation to be submitted for publication. In this chapter, the repair of UV damage DNA is examined in untreated RIF cells, as well as RIF cells treated with Photofrin mediated PDT, JM2929 mediated PDT, UV, cisplatin and hyperthermia. These chapters are followed by a summary chapter of all work, and a complete reference list. All work presented in this thesis has been carried out by the author.

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This work is dedicated to my partner Sean and to my family for their support, encouragement, and supply of dark chocolate.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. A. Rainbow for teaching me science. Without his encouragement and patience this work would not have been possible. I would also like to thank Dr. Singh, the other member of my committee. I would like to give special thanks to Bruce McKay and Otto Sanchez-Sweatman for their academic insight and guidance. I would also like to thank Murray Francis and Todd Bulmer for their assistance and humor in the lab. To Tamie Poepping and Deidre Batchelar, I give my deepest gratitude for their friendship, laughter and support.

I would like to thank Sunera Thobani, Shree Mulay, Laura Sky, and Dr. Boetzkes for encouraging me to pursue my master's degree in science. I thank my brother and my sister for being by my side, and to my parents, I give my thanks for instilling in me the drive to achieve. And lastly, I thank my partner Sean for listening to me type over the phone.

CHAPTER 1

INTRODUCTION

INTRODUCTION

A. Cancer Treatment

Cancer represents aberrations in cellular behavior involving many aspects of molecular biology. In some cases, cancerous cells continue to multiply when normal cells would be quiescent. Some cancer cells invade surrounding tissues and spread through the body to form secondary areas of growth in a process called metastasis (Darnell et al. 1990). Common cancer treatments in use today such as ionizing radiation and chemotherapy are not always ideal because sometimes these therapies do not discriminate between normal cells and cancerous cells. Also, tumor cells can become resistant to physical and chemical therapeutic agents that damage normal cells instead (Moossa et al. 1991). A relatively new targeted cancer treatment approach uses the activation of photosensitive compounds that selectively concentrate in tumor tissues (Lowdell 1994, Manyak et al. 1988, Pass 1993). This cancer therapy, known as Photodynamic Therapy (PDT), is a relatively selective and local treatment modality. PDT mediated by the haematoporphyrin derivative Photofrin has been undergoing Phase III clinical trials in Canada, U.S.A., Japan, and some countries in Europe, for the treatment of skin, breast, and bladder cancers (Dougherty 1993, Dougherty and Marcus 1992).

B. Photodynamic Therapy

B.1. Development of PDT

In 1900, Raab discovered that microorganisms were destroyed when treated with acridine dyes in the presence of visible light (Moan et al. 1979). The first oncologic use of PDT came with the combination of eosin and light to treat skin cancer in 1903. In 1911, Hausman's initial experiments with haematoporphyrin introduced the concept of photoinduced cellular cytotoxicity for use in medicine (Pass 1993). Since then, the porphyrin based sensitizer has been a popular model for photosensitizers used in PDT. Prior to 1970, there were scattered reports of clinicians attempting to treat breast and bladder cancers using haematoporphyrin derivative (HpD), with some success. It was not until after 1970 when Dougherty and his group at Roswell Park described their first sustained series of studies investigating the mechanisms of PDT action in human and animal malignancies, that the field of PDT research began to grow (Wieman and Fingar 1992). To date, there have been more than 10,000 patients treated with PDT (Berg et al. 1995). A variety of different types of tumors have been shown to respond to PDT such as breast cancer, B-cell carcinomas, skin cancer, and bladder cancer (Dougherty 1993). However, PDT is efficient only in cases where the entire tumor can be reached by light. For this reason, tumors thicker than 5-7 mm are rarely completely eradicated by PDT unless there is intrinsic light exposure using fibre optics (Berg et al. 1995).

B.2. Mechanisms of Photosensitization

B.2.i. Type I Photosensitization

The Type I mechanism of photodynamic action involves the direct transfer of energy by the excited triplet state of photosensitizer to either substrate or solvent (Dougherty and Marcus 1992, Gomer et al. 1989, Henderson and Dougherty 1992). The Type I reaction occurs when photosensitizers in their ground state absorb light energy and are excited into a triplet state via a short lived single state. The excited triplet state of photosensitizer molecules act either directly on biological molecules or on solvent, by hydrogen atom, or by electron transfer to form radicals and radical ions. (Henderson and Dougherty 1992). These radicals can then further react with oxygen to create oxygenated products such as hydrogen peroxide, hydroxyl radicals and/or superoxide ions. Although the Type I photosensitization mechanism is thought to contribute a minor role in PDT mediated cytotoxicity, it may be directly involved in cellular damage depending on sensitizer, substrate, and oxygen concentrations, as well as on sensitizer binding properties (Gomer et al. 1989).

B.3.ii. Type II Photosensitization

Following photosensitizer delivery to tumor cells and activation by light, the excited triplet state of photosensitizer can undergo energy transfer with the ground state of molecular oxygen to form singlet oxygen, which is a highly reactive oxidative species. The singlet state of oxygen can then further react with surrounding biomolecules (Pass 1993). The Type II photosensitization reaction is thought to be more

prevalent within cells due to the abundance of water (Gomer et al. 1989, Dougherty and Marcus 1992).

B.3. Requirements for Photodynamic Therapy

B.3.i. Photosensitizers

There are four basic properties that make a sensitizer useful for photodynamically induced cytotoxicity: i) selective retention or uptake by tumor cells; ii) high quantum yield for the generation of Type I or Type II photochemical reactions; iii) significant absorbance at wavelengths above 600 nm; iv) and photolability (Pass 1993, Moan et al. 1979, Gomer et al. 1989).

B.3.i.i. Selective Uptake and Retention by Tumors

Ideal cancer therapies target anticancer agents to cancer cells alone, and avoid the destruction of normal cells. Investigators have used fluorescence and radiolabelling techniques to follow photosensitizer accumulation in vivo and in vitro (Henderson and Dougherty 1992). For reasons not fully understood, PDT photosensitizers have shown the property of preferential accumulation in tumors during in vivo treatment (Lowdell 1994, Manyak et al. 1988, Pass 1993). It has been suggested that the decreased pH of tumor cells makes sensitizers to become more water soluble and therefore selectively retained by tumor tissue (Pass 1993). Tumor vasculature is also thought to contribute to sensitizer accumulation. Poor lymphatic circulation in the tumor vasculature, leading to the aggregation of sensitizer molecules, is thought to contribute to

preferential sensitizer accumulation (Henderson and Dougherty 1992, Dougherty and Marcus 1992, Dougherty 1993, Gomer et al. 1989).

B.3.i.ii. High Quantum Yield

The ratio of photons emitted to the number of photons absorbed by a system (in this case photosensitizer) is known as quantum yield (Cantor and Schimmel 1969). The excitation of the sensitizer into its triplet state from its ground state (singlet state) by the absorption of light results in either Type I or Type II photochemical reactions. Most photosensitizing compounds have heterocyclic ring structures that are capable of capturing light energy in the form of photons and then transferring that energy in the form of an electron to other molecules (Weiman and Fingar 1992). The quantum yield for the triplet excited state is usually high (greater than 0.7) for efficient photosensitizers (Gomer et al. 1989).

B.3.i.iii. Sufficient Tissue Penetration

Light fluence (number of photons per unit area) in tissue decreases exponentially with distance (Henderson and Dougherty 1992). The wavelength of light that excites photosensitizer molecules varies according to properties of the photosensitizer used. Longer wavelengths of light result in increased penetration depths in tissue. PDT induced cytotoxicity can occur in deeper tissues when the sensitizer being used is excited at longer wavelengths of light. Most recently, Pandey et al. (1996) have found a class of compounds made of carbodiimide analogs of

bacteriochlorins prepared from the corresponding bacteriopurpurin esters that act as photosensitizers with strong absorption at 800 nm (Pandey et al. 1996). It is a goal in PDT research to design the most suitable photosensitizer that is activated at longer wavelengths for increased tissue penetration.

B.3.i.iv. Photolability

Photolability is the rapid clearance of photosensitizer from normal tissue. Ideally, once photosensitizer molecules have undergone Type I or Type II photochemical reactions in tumor tissue, they should lose their cytotoxic potential. Photosensitizers should be easily degraded in the body to avoid lingering photosensitivity in patients. Photofrin, which is currently undergoing Phase III clinical trials has poor photolability (Agarwal et al. 1992, van Leengoed et al. 1993). Often patients treated with Photofrin mediated PDT (Ph-PDT) experience increased skin sensitivity for up to 2 months following treatment (Amato 1993).

B.3.ii. Light Sources

The second requirement for PDT is a light source to provide excitation of photosensitizer molecules. It is necessary to bring the photosensitizer activating light to the target tissue. Typical photosensitizers used in PDT have absorbances between 580nm-700nm. Conventional wavelength filtered lamps can be used for PDT. However, for more accurate delivery, lasers are used for the emission of a monochromatic form of intense collimated light energy (Manyak et al 1988). Argon

pump-dye lasers exciting Kiton red or rhodamine B to produce red light are often used. These lasers can also be coupled to fibre optic cables for delivery (Pass 1993).

B.3.iii. Oxygen

During the photodynamic process, molecular oxygen is transformed into singlet oxygen ($^1O_2$), which has very strong oxidative properties (Cannistraro et al. 1982, Dougherty et al. 1976, Gomer and Razum 1984, Weishaupt et al. 1976). Singlet oxygen species are produced when the excited triplet state of photosensitizer molecules transfer their energy to ground state oxygen. The lifetime of singlet oxygen is approximately 1μs in tissue (Moan et al. 1979), 4μs in water, 50-100μs in lipid, and 0.6μs in a cellular environment (Henderson and Dougherty 1992). The distance of diffusion of singlet oxygen in the cellular environment has been approximated to 0.1 μm (Moan et al. 1989). The electrophilic nature of singlet oxygen is well suited for producing oxidized forms of surrounding biomolecules by reacting with the electron rich regions of these molecules (Gomer et al. 1989). The absolute requirement for oxygen in the photosensitizing action of clinically used porphyrins has been documented in solution, in culture and in vivo (Pass 1993, Gomer et al. 1988). However, Kostron et al. (1988) have reported contradicting evidence suggesting that high concentrations of HpD resulted in a different pathway of generating cytotoxic oxygen radicals through a direct reaction of activated HpD with the tumor cell, without the requirement for molecular oxygen (Kostron et al. 1988).

B.4. Types of Photosensitizers

There are now many classes of photosensitizers available for PDT use. A few of these include porphyrin derivatives, benzoporphyrin derivatives, phthalocyanines such as aluminum, zinc and ruthenium phthalocyanine which are sulfonated to varying degrees, and nile blue derivatives. As mentioned previously, Pandey et al. (1996) have recently found a class of compounds made of carbodiimide analogs of bacteriochlorins prepared from the corresponding bacteriopurpurin esters that act as photosensitizers with strong absorption at 800 nm. (Pandey et al. 1996). Those derived from the haematoporphyrin molecule are generally referred to as "first generation" sensitizers. The most popular photosensitizer that is currently in Phase III clinical trials is the purified form of hematoporphyrin derivative (HpD), referred to as Photofrin. Sensitizers such as phthalocyanines and cationic dyes used for PDT are referred to as "second generation" sensitizers. Two classes of photosensitizers, namely porphyrins and phthalocyanines are discussed in further detail below.

B.4.i. Porphyrin sensitizers

A mixture of monomeric and aggregated porphyrins was produced in 1961 by first acetylating and then reducing crude haematoporphyrin (Hp). This product was named haematoporphyrin derivative (HpD)(Gomer et al. 1988). Since then, many types of porphyrin based sensitizers have been developed for photodynamic use (Pandey et al. 1989). Progress in PDT clinical research has developed on the basis of studies involving mostly HpD derivatives including Photofrin. Moan et al. (1987)

reported on a series of haematoporphyrin diamyl ethers and observed that photosensitizer induced cytotoxicity increased with decreased sensitizer polarity (Pandey et al. 1989). A study conducted by Woodburn et al. (1992) on 15 different porphyrin sensitizers showed a correlation between subcellular localization and degree of phototoxicity, with the three most effective porphyrins all having cationic side chains, and all three localizing in mitochondria (Woodburn et al. 1992). Possible explanations for preferential mitochondrial retention of such sensitizers include mitochondrial membrane potentials, pH, benzodiazepine receptors, and lipophilicity (Woodburn et al. 1992). Photofrin is a mixture of non-metallic oligomeric hexar derivatives of haematoporphyrin units that are linked together through ether or ester bonds (Dougherty and Marcus, 1992.). The oligomers range in size from 2-8 porphyrin units, although the major portion appears to be trimeric (Fig. 1A). Prolonged photosensitivity and a low extinction coefficient (absorbance of radiant energy of a substance in $M^{-1}cm^{-1}$) (Harris and Kratochvil 1981) are considered as drawbacks of Photofrin (van Leengoed et al. 1993). For this reason new HpD derivatives and other classes of sensitizer molecules are being investigated for use in PDT.

B.4.ii. Phthalocyanines

Phthalocyanines (Pc's) are non-toxic and have high extinction coefficients in the red region of the spectrum (Ben Hur et al. 1987). Pc excitation occurs between 670-680nm. Pc's are structurally similar to porphyrins but are complexed with

diamagnetic metal ions such as Ruthenium, Zinc or Aluminum. The type of metal ion chelated within a Pc has a considerable impact on the tumor retention efficiency of the photosensitizer (Agarwal et al. 1992). The metal ion also increases the quantum yield and lifetime of the photosensitizer's excited triplet state. However, the metal ion makes unsubstituted photosensitizer molecules more hydrophobic and liable to aggregate in aqueous solutions (van Leengoed et al. 1993). For this reason, sulfonate groups are added to Pc's to reduce this tendency. The degree of sulfonation has been implicated in the extent of vascular damage seen in vivo. There is an inverse relation between sulfonation number and the extent of fluorescence localization of photosensitizer in sites vital to cell survival (van Leengoed et al. 1993). Like porphyrins, phthalocyanines accumulate in membrane fractions. Photosensitization by Pc's has been shown to cause damage in the plasma membrane as well as in the membranes of subcellular organelles such as the mitochondria (Evans et al 1989). PDT mediated by a novel Pc, Ruthenium (II) Pc-bis-(triphenyl-*m*-monosulfonate) potassium salt (referred to as JM2929), is examined in the present work. JM2929 is a water soluble sensitizer which is activated at 650 nm (Fig.1B).

B.5. Photosensitizer Localization and PDT Induced Damage

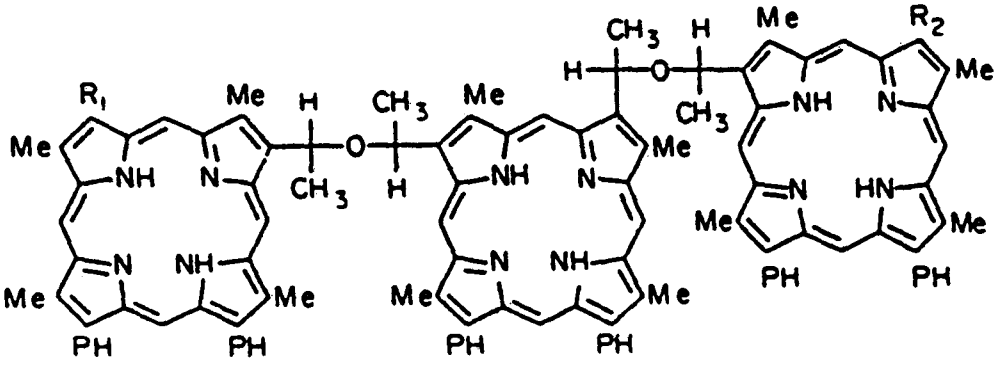
Intracellularly, the plasma membrane (Kessel et al. 1977, Moan et al. 1989), mitochondria (Berns et al. 1982, Boegheim et al. 1988, Hilf et al. 1987, Singh et al. 1991), lysosomes (Gomer et al. 1988, Sasaki et al. 1993, Torinuki et al. 1980), endoplasmic reticulum (Moan et al. 1989), and DNA in the nucleus (Dubbelman et al.

Figure 1: Structure of Photofrin and JM2929 photosensitizers

The major component of Photofrin (A) is the porphyrin trimer. The R_1 group represents $CH(OH)CH_3$ and the R_2 group represents $CH=CH_2$. PH groups represent $(CH_2)2COOH$. (From Dougherty and Marcus 1992).

The pthalocyanine ring of JM2929 (B) with a central Ruthenium (Ru) atom. Ligand (L) represents (triphenylphosphine-m-monosulfonate) potassium salt.

A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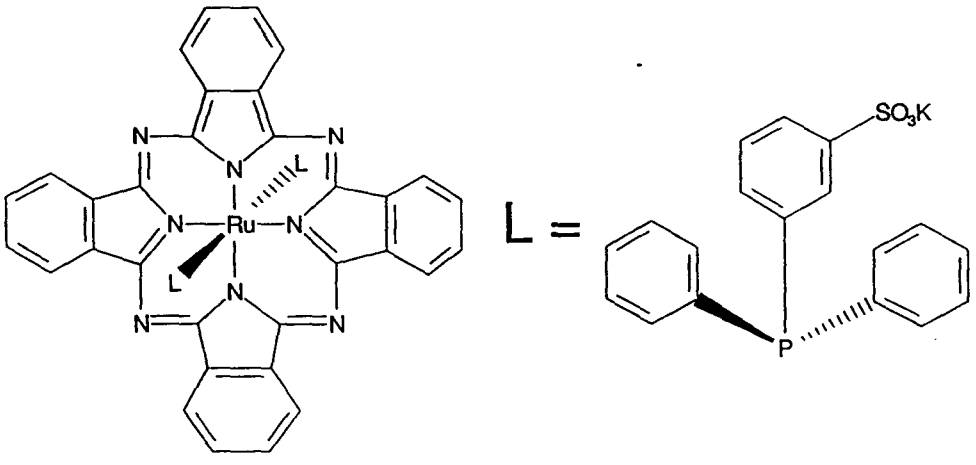
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1982, Crute et al. 1986, Gomer et al. 1988), have been shown to be targets for photosensitizer localization. Methods used to detect intracellular photosensitizer localization include subcellular fractionation and fluorescence microscopy (Moan et al. 1989). Lipophilic sensitizers generally localize in the membrane structures, whereas hydrophilic sensitizers accumulate in lysosomes (Moan et al. 1989). There is also evidence to suggest photosensitizer transport by serum proteins. Albumin delivers bound sensitizers to the vascular stroma (Obochi et al. 1993). Lipoproteins, especially low density lipoproteins, help to internalize photosensitizers into malignant cells that have large number of LDL receptors on their cytoplasmic membrane (Obochi et al. 1993).

B.5.i. Tumor Cells and Tumor Vasculature

Photosensitizer retention is higher in tumor tissue compared to normal tissues such as skin, muscle, brain and lungs (Henderson and Dougherty 1992). There may be several reasons for preferential accumulation of sensitizer in tumor tissue. Hamblin et al. (1994) suggest the following possibilities: (i) neoplastic cells have an increased number of low density lipoprotein (LDL) receptor sites, which is a common feature of other rapidly dividing cells (due to their increased requirements for cholesterol for membrane biosynthesis); (ii) decreased intratumoral pH in tumors may affect solubility and retention of photosensitizer molecules; (iii) tumor cell membranes may be more hydrophobic than membranes of normal cells, leading to increased accumulation of hydrophobic sensitizers; (iv) and infiltration of tumors by tumor associated

macrophages which contain up to nine times the concentration of photosensitizer compared to normal cells (Hamblin et al. 1994).

There is also substantial evidence to suggest that the tumor vasculature plays a crucial role in, but is not solely responsible for, the tumor response following PDT in vivo. Poor lymphatic drainage and increased vascular permeability leading to sensitizer accumulation of sensitizer may contribute to the selective retention of photosensitizers in tumors (Gregory Roberts and Hasan 1993). Tumor blood flow has been shown to be significantly reduced following PDT treatment (Gomer et al 1988). Histological examinations have demonstrated destruction the of the vascular endothelium following PDT in vivo. Henderson et al. (1985) showed that microscopically, the first signs of tumor damage were the congestion of tumor blood vessels and the extravasation of erythrocytes (Henderson et al. 1985). Damage to blood vessels directly inhibits proper blood flow to tumor tissue, leading to insufficient oxygen circulation to tumor cells. The loss of oxygen may contribute to tumor cell death. van Geel et al. (1996) showed that in Ph-PDT treated radiation induced fibrosarcoma (RIF-1) tumors, more than half of the total tumor weight became hypoxic compared to only 4% of the tumor weight in untreated control tumors (van Geel et al. 1996). However for the RIF-1 tumor, despite histological evidence suggesting severe vascular damage following hyperthermia, tumors have been shown to recover. Similar vascular damage is also caused by PDT in these tumors (Henderson et al. 1985). This result suggests that vascular damage alone does not cure RIF-1 tumors, and that in vitro studies of RIF-1 cells are of relevance.

B.5.ii. Subcellular Targets

B.5.ii.i.Plasma Membrane

Plasma membranes are mainly composed of a phospholipid bilayer, proteins which are embedded in the bilayer, and carbohydrates that may be linked to the lipids and proteins. Fatty acid chains in phospholipids can either be saturated or unsaturated (Stryer 1988). Short porphyrin incubation time periods such as less than one hour, followed by light treatment primarily lead to plasma membrane damage (Gomer et al. 1988). Kessel (1977) found that the initial site of photoactivated porphyrin toxicity was at or near the plasma membrane in murine leukemia L1210 cells (Kessel 1977). Unsaturated fatty acid chains have been shown to be photooxidized by singlet oxygen that is produced by the Type II PDT mechanism (Gomer et al. 1988). Lipid peroxidation as well as protein crosslinking in plasma membranes have also been observed in mammalian cells following PDT treatment (Gomer et al. 1988). When cells are illuminated at an early stage following photosensitizer treatment, electron micrographs show a cellular swelling effect that is thought to be due to an influx of water caused by damage to the plasma membrane (Moan et al 1989). Plasma membrane damage can lead to inhibited membrane transport of amino acids and nucleosides (Gomer et al. 1988).

B.5.ii.ii. Mitochondria

Mitochondria are specialized organelles where ATP synthesis and oxidative phosphorylation processes occur that are necessary for normal cell function.

Mitochondria synthesize heme, lipids, amino acids and nucleotides, and mediate the intracellular homeostasis of inorganic ions (Schatz 1995). Lipophilic, or hydrophobic sensitizers that target the mitochondrial membrane affect critical mitochondrial enzymes such as succinate dehydrogenase and cytochrome c oxidase (which are involved in oxidative phosphorylation and electron transport). Such enzymes include succinate dehydrogenase and cytochrome c oxidase. Cellular ATP levels have also been shown to decrease following photodynamic treatment (Ricchelli et al. 1993). Reduction in ATP levels is caused by damage to inner membrane mitochondrial carriers, (especially the ADP/ATP translocator) by photoinduced oxidation of thiol groups of the active site (Ricchelli et al. 1993). Boegheim et al. (1988) reported increased mitochondrial membrane potential in HpD-PDT treated L929 cells attributed to direct or secondary inhibition of the ATP-synthetase (Boegheim et al. 1988). Decreased oxygen consumption rates and ATP level following PDT was also observed in L929 cells. V-79 Chinese hamster cells treated with Ph-PDT showed light dose dependent decreases in both succinate dehydrogenase and cytochrome c oxidase activities (Singh et al. 1987). Morphological and functional differences between the mitochondria of Ph-PDT sensitive and Ph-PDT resistant RIF cells have been suggested to be responsible for differences in their sensitivity to Ph-PDT (Sharkey et al. 1993).

B.5.ii.iii. Lysosomes

Lysosomes are responsible for the degradation of proteins, nucleic acids and lipids. Lysosomes contain degradative enzymes such as phosphatases, nucleases, and

proteases. In 1980, Torinuki et al. reported that levels of lysosomal enzymes released into the supernatant of lysosomal fractions were significantly high when irradiated in the presence of HpD (Torinuki et al. 1980). Torinuki et al. (1980) suggested that lipid peroxides formed in lysosomal membranes following PDT damage resulted in increased permeability and destruction of the lysosomal membrane.

B.5.ii.iv. Nuclear DNA

Once a cell has lost its plasma membrane structure and cytoplasmic leakage has occurred, the effects of nuclear DNA damage may be of less importance. In 1981, Christensen reported that photosensitization caused an irreversible division delay at mitotic metaphase in some cells that did not suffer from lysis of the plasma membrane. This suggested a possible role for DNA damage in cell killing (Blazek and Hariharan 1984). DNA is readily damaged by reactive oxygen species such as hydroxyl radicals (Hall 1994) which can be produced during the photosensitization process. There are many investigators who have found that nuclear DNA is a subcellular target in photodynamic damage by both porphyrins and phthalocyanines (Gomer 1980, Evans et al. 1989, Kvam and Stokke 1994, Blazek and Hariharan 1984, Fiel et al. 1981). However the range of DNA damage elicited by photosensitization is limited for several reasons. The first limitation is the short diffusion range of photosensitizer molecules (Moan et al 1989, Kvam and Stokke 1994). For membrane bound sensitizers, only DNA that is localized at this distance to the nuclear membrane is likely to be affected by singlet oxygen produced by photodynamic action. For hydophilic sensitizers that are

able to enter the nuclear membrane, there is evidence of photosensitizer accumulation in rings around the nuclei (Kvam and Stokke 1994). At the level of the nucleus, single stranded breaks and alkali-labile sites in DNA, sister chromatid exchanges, and chromosome aberrations can be caused by PDT (Gomer et al. 1988, Gomer 1980). Results suggest that the selective degradation of the guanine moiety of DNA by photosensitization of porphyrins results in the generation of single stranded segments of DNA (Gomer 1980, Dubbelman et al. 1982). In 1987 Ben Hur et al. reported that in V79 Chinese hamster cells treated with Pc plus light, there was a smaller yield in DNA lesions (measured as single strand breaks via alkaline elusion) compared to the amount caused by an equitoxic dose of gamma radiation. However there was a greater yield in DNA-DNA cross links and DNA-protein cross links produced in V79 cells following treatment with Pc and light, compared to gamma-radiation treatment (Evans et al. 1989). Evidence exists for mutagenic effects on mouse lymphoma cells (L5178Y) treated with Pc mediated PDT at the thymidine kinase locus (Evans et al. 1989). Also, after treatment with tetra(3-hydroxyphenyl) porphyrin plus light, V79-379A cells were shown to be mutagenic at the hypoxanthineguanine phosphoribosyl transferase (hprt) locus (Noodt et al. 1993).

B.6. PDT induced Stress Proteins and affected Genes

A number of stress proteins and genes involved in other oxidative stress responses have been reported to be influenced by the photodynamic damage process. Reactive oxygen species produced by photodynamic reactions initiate oxidative damage

on many types of biomolecules. Particularly, hydroxyl radicals are quite reactive on proteins, causing covalent cross linking, fragmentation, and modification of almost all amino acid residues. This results in the ultimate loss of protein function and increased protein susceptibility to degradation by proteolytic enzymes following PDT (Prinze et al. 1990). Examples of proteins and genes affected include elevated levels of the glucose regulated protein (GRPs) family following porphyrin mediated PDT (Gomer et al. 1991); induction of a family of heat shock proteins (HSPs) using benzoporphyrin derivative (Anderson et al. 1989); induction of alpha-2 microglobulin receptor expression (Luna et al. 1994); induction of heme oxygenase expression (Gomer et al. 1991b); and increased expression of a set of early response genes including c-fos, c-jun, c-myc and egr-1 (Luna et al. 1994). Increased expression of these proteins and genes have been studied in different cell lines, following the PDT effects of different photosensitizers. Another type of stress response reported is the increase in cytoplasmic free calcium following photodynamic action. Penning et al. (1992) have postulated that the transient increase of calcium seen in both Chinese Hamster Ovary cells immediately following aluminum phthalocyanine mediated PDT, and in T24 human bladder carcinoma cells following HpD mediated PDT, may contribute to increased survival of treated cells (Penning et al. 1992).

C) Cell lines commonly used for PDT studies in vitro

In vitro studies of PDT resistant cells derived from PDT sensitive lines have contributed to the understanding of the cellular targets and biochemical effects involved in PDT cytotoxicity. Common cell lines studied in vitro include the parent and derivatives of RIF-1 cells, the Chinese Hamster Ovary (CHO) cell line, the SMT-F murine mammary carcinoma cell line, the EMT-6 (experimental murine mammary tumor) cell line and the L5178Y mouse lymphoma cell line. Parental cells and variant strains of RIF cells are discussed in further detail below.

C.i. RIF-1 parent cell line

It is advantageous to have a tumor model system that is sustained both in vivo and in vitro. The RIF tumor, commonly used in PDT research, grows both in vivo and as clones in vitro, is minimally immunogenic, and does not produce early spontaneous metastasis (Twentyman et al. 1980). This tumor model system was developed by Twentyman et al. in 1980, by the delivery of 40 fractions of 250 kV X-radiation, 400 rads/fraction, over a 12 week period, on the hindlimb of a male C3H/Km mouse from an inbred colony. Multiple RIF tumors were pooled into a single cell suspension. The cells were grown in monolayer and injected back in the flank of mice to form tumors for three cycles. The final stock culture was named RIF-1.

The plating efficiency of the solid RIF-1 tumor is reported to be 24.2% with a standard deviation of 4.7%. The in vitro doubling time is approximately 14 hours. The

in vitro plating efficiency for fed plateau phase RIF-1 cells is approximately 69%. Two karyotypes in RIF-1 cells, namely 40 and 80 chromosomes are seen, making it a diploid and tetraploid tumor (Twentyman et al. 1980). The RIF-1 cell line has been shown to be responsive to photodynamic damage mediated by Photofrin (Singh et al. 1991, Luna and Gomer 1991).

C.ii. Ph-PDT resistant RIF-8A cells

Singh et al. (1991) developed a cell line with some degree of resistance to Ph-PDT (Singh et al. 1991). The resistant strain (RIF-8A) was derived by harvesting resistant colonies of RIF-1 cells treated with varying doses of Ph-PDT (Singh et al. 1991). The level of Photofrin uptake per unit cell volume was no different in the RIF-8A cells compared to the parent RIF-1 cells, and resistance to Ph-PDT could not be attributed to classical multidrug resistance (Singh et al. 1991). The RIF-8A strain has been characterized by Sharkey et al. (1992) to have distinct morphological and functional differences from the parent RIF-1 cell line (Sharkey et al. 1992, Sharkey et al. 1993). RIF-8A cells are larger than the RIF-1 parent cells and the RIF-8A karyotype appears inconsistent. Most commonly, polyploidies of 120 chromosomes are seen (Sharkey et al. 1993). The RIF-8A mitochondria are smaller, stain more densely and display a higher cristae density compared to the mitochondria of RIF-1 cells. Sharkey et al. (1992) also reported increased ATP amounts and succinate dehydrogenase activity in the RIF-8A strain, whereas the rates of oxygen consumption were reported to be similar between RIF-8A and RIF-1 cells. Differences in ATP production between RIF-1

and RIF-8A cells may be due to a significantly higher susceptibility to inhibition of glycolytic activity in RIF-1 cells compared to RIF-8A cells following Ph-PDT (Sharkey et al. 1993). Sharkey et al. (1993) have suggested that mitochondrial differences are responsible for the PDT resistance seen in the RIF-8A cells (Sharkey et al 1993).

Further studies have shown that the RIF-8A strain is cross resistant to cisplatin (Moorehead et al. 1994), which is an anticancer agent that causes damage through DNA adduct formation (Freidberg et al. 1994). It was concluded by Moorehead et al. (1994) that a decrease in the plasma and/or mitochondrial membrane potentials provides the RIF-8A strain with a survival advantage. Interestingly, another cisplatin sensitive cell line 2008 (human ovarian carcinoma), and its cisplatin resistant C13 derivative show mitochondrial differences that are similar to the mitochondrial differences seen between RIF-8A and RIF-1 cells. As well, C13 cells show cross resistance to Ph-PDT (Sharkey et al. 1993). Cross resistance of the RIF-8A cells to UV light (which causes pyrimidine dimers and 6-4 photoproducts in DNA) has also been reported (Di Prospero et al. 1996, Freidberg et al. 1994).

C.iii. RIF-1G, RIF-P16, and CPR-C1 cells

Ph-PDT resistant cells developed by Luna and Gomer (1991) were derived from RIF-1G cells (same parental cell line as RIF-1) by 10 cycles of Ph-PDT treatment following a 16 hour photosensitizer incubation period prior to each treatment (Luna and Gomer 1991). Two resistant clones RIF-P16CL4 and RIF-P16CL8 were derived by this method. Photofrin uptake in both RIF-P16 variants was found to be similar to

parental RIF-1G cells (Luna and Gomer 1991). Both RIF-P16 variants exhibited modest resistance to ionizing radiation (at a dose range of 15Gy). RIF-P16CL4 cells demonstrated increased sensitivity to hyperthermia. The CPR-C1 strain, with increased resistance to Ph-PDT compared to RIF-P16CL8 cells, was developed by isolating surviving colonies of RIF-P16CL8 cells exposed to 6 more Ph-PDT cycles (Luna personal communication).

D. In vitro sensitivity of cells to PDT

In vitro sensitivities of a number of mammalian cell lines following PDT have been examined in many ways including measurement of differences in cell survival, mitochondrial damage, lipid synthesis/fatty acid uptake, and DNA damage. For example, cell survival following PDT has been measured by clonogenic assays (Singh et al. 1991, Luna et al. 1991), viable cell count by Coulter counter (Berns et al. 1982), and ^{51}Chromate release assay (Biade et al. 1992). Intracellular binding of photosensitizer to mitochondria seen by fluorescence techniques has been correlated with HpD-PDT mediated growth inhibiting effects in rat kangaroo epithelial cells and normal mouse embryonic fibroblasts (Berns et al. 1982). Delocalization of Rhodamine 123 resulting from the dissipation of the electrochemical gradient has been reported in Ph-PDT damaged mitochondria of V-79 Chinese hamster cells (Singh et al. 1987). Reduced uptake of oleic acid in photosensitized human fibroblasts suggests that inhibition of membrane phospholipid synthesis plays an important role in the phototoxic effect of Photofrin (Biade et al. 1992). Alkaline elution method has been

used to detect DNA strand breaks in Chinese hamster cells treated following HpD-PDT (Blazek and Hariharan 1984).

Not all cell types appear to have similar PDT sensitivities. Studies have identified a number of cell lines with a defined biochemical defect to show varied PDT response (Di Prospero 1994). Human melanoma cell lines have been shown to have differences in their sensitivity to porphyrin mediated PDT (James et al. 1994). Some cells with multi-drug resistant (MDR) phenotype such as chinese hamster ovary MDR cells have shown cross-resistance to Photofrin or HpD mediated PDT (Singh et al. 1991). Also, the Chinese hamster ovary (CHO)-AUXB1 cell mutant, deficient in folypolyglutamate synthetase enzyme, shows cross sensitivity to UV light and Ph-PDT. LY-R murine leukemia cells which are sensitive to UV damage, have been reported to have a reduced ability to carry out the incision step as well as the repair replication step of nucleotide excision repair (NER) following UV exposure. LY-R cells have also shown cross-sensitivity to chloroaluminum Pc-PDT and Ph-PDT suggesting a correlation between NER and PDT sensitivity (Evans et al. 1989, Ramakrishnan et al. 1989). However, Gomer et al. (1988) were unable to see a difference in sensitivity between normal fibroblasts and those from patients suffering form xeroderma pigmentosum (XP) and ataxia telangiectasia (AT) (which are DNA repair deficiency syndromes) following Ph-PDT (Gomer et al. 1988). Similar clonogenic survival following HpD-PDT was also seen for fibroblasts from normal, XP, and Fanconi's anemia (FA) (rare autosomal recessive DNA repair disease) patients (Noncentini 1992). These results suggest that the incision step of nucleotide excision repair which is deficient in XP cells, as well as

the gene products that are deficient in AT and FA cells, are not involved in the mechanism(s) responsible for cell survival following PDT (Di Prospero et al. 1996). Excision repair in murine L929 fibroblasts treated with HpD mediated PDT was reported to be severely inhibited at a stage beyond the incision step (Boeigheim et al 1987). It is suggested that there may be some overlap in the pathway required for repair of PDT damage and the NER pathway beyond the incision step (Di Prospero et al. 1996, Boegheim et al. 1987). Cross resistance of Ph-PDT resistant RIF-8A cells to UV suggests that compared to parental RIF-1 cells, RIF-8A cells may have enhancement of this overlapping section of the NER pathway (Di Prospero et al. 1996).

E. Use of Viral Probes to examine sensitivity to and repair of damage from PDT and other agents in vitro

E.1. Viral Capacity Assay

The viral capacity assay can be used as an in vitro test for the sensitivity of cells following drug treatments (Parsons et al. 1986). The viral capacity measures the ability of cells, which have been treated with a chemical or physical agent, to support viral growth (Di Prospero et al. 1996, Bockstahler et al. 1982). In the viral capacity assay, cells are treated before or during infection with untreated virus. Virus replication is inhibited by agents which affect any cellular function needed by the virus to replicate such as DNA, RNA, or protein synthesis (Parsons et al. 1989). Inactivation of the cellular capacity to support viral replication can be used to measure the sensitivity of tumor cells to a variety of host cell damaging agents (Parsons et al. 1989). Agents such

as UV, gamma rays, proflavin plus visible light, and 8 methoxypsoralen plus UV light have been shown to decrease cellular capacity to support viral infection (Di Prospero et al. 1996, Bockstahler et al. 1982). The results of a delayed capacity assay suggest the increased resistance of RIF-8A cells to PDT and UV results from elevated levels of repair of UV and PDT damage in RIF-8A cells compared to RIF-1 cells (Di Prospero et al. 1996).

E.2. Host Cell Reactivation Assay

Host cell reactivation (HCR) of virus is a sensitive and quantitative measure of the repair capacity of the host cell, not treated with a damaging agent, to repair and hence replicate damaged viral DNA induced by a number of physical and chemical agents (Parsons et al. 1986, Day et al. 1975, Rainbow 1989). HCR of irradiated adenovirus has been used by a number of investigators to detect DNA repair deficiencies in fibroblasts from cancer prone individuals (Day et al. 1974, Rainbow 1980, Rainbow 1989). In the past, measurement of HCR of virus has depended on either scoring for viral plaque formation (Day 1981), or detection of viral antigens by immunofluorescence microscopy (Rainbow 1980). More recently, DNA repair has been determined by measuring HCR of damaged reporter genes such as *lacZ* (β-galactosidase) or *cat* (chloroamphenicol acetyl-transferase) expressed by recombinant nonreplicating human adenovirus vectors used to infect host cells (Valerie and Singhal 1995, McKay and Rainbow 1996).

E.3. Enhanced Reactivation of virus

Enhanced reactivation (ER) of virus is a third approach involving the combination of HCR of damaged viral DNA in cells that are pretreated with damaging agent (Parsons et al. 1986). Pretreatment of cells with UV, γ-irradiation or heat shock have been shown to enhance reactivation of DNA damaged virus in mammalian cells (Coppey and Menezes 1981, Dion and Hamelin 1987, Jeeves and Rainbow 1983, Lytle and Carney 1988, McKay and Rainbow 1996). It has been suggested that ER of UV-damaged DNA virus in mammalian cells results from an inducible DNA repair pathway (Rainbow 1981). ER results of UV-damaged reporter genes following heat shock (McKay and Rainbow 1996) and UV (Francis and Rainbow 1995) pretreatment to mammalian cells also provide support for an inducible DNA repair pathway.

F. PDT in Combinational Therapies

The potential exists for combinational therapies involving PDT. The basic requirements of PDT namely sensitizer, light and oxygen are easily integrated into many other anticancer therapies such as ionizing radiation or hyperthermia.

F.1. PDT and Hyperthermia

In 1967, Cavaliere et al. reported on the selective toxic effect of elevated temperatures on tumor cells (Cavaliere et al. 1967). Overgaard has reviewed the selective destruction of malignant cells in vivo following hyperthermia (Overgaard 1977). Effects following hyperthermia treatment seen in both normal and malignant

cells include cellular changes in RNA synthesis, DNA and protein synthesis, cell cycle, glycolysis, and lysosomal function.

Many investigators have reported on the interaction of hyperthermia and photodynamic therapy (Christensen et al. 1984, Berns et al. 1984, Waldow et al. 1987, Waldow et al. 1984, Waldow and Dougherty 1984, Waldow et al. 1985, Melloni et al. 1984, Svaasand 1985, Henderson et al. 1985). Heat treatment following either porphyrin or phthalocyanine mediated PDT results in synergy (increased cell kill following the combination of two treatments compared to cell kill resulting from each treatment alone) (Glassberg et al. 1991, Mang 1990, Christensen et al. 1984, Henderson et al. 1985). Although similarities can be seen immediately following independent PDT and hyperthermia treatments in vivo, Henderson et al. (1985) suggested that these two modalities lead to tumor destruction by different mechanisms (Henderson et al. 1985). In vitro, PDT and hyperthermia treatments share similar subcellular targets such as the plasma membrane. Both treatments also lead to protein denaturation (Henderson et al. 1985). It has been shown that Ph-PDT induces a series of heat shock proteins (hsp) including hsp70, and hsp32 (Curry and Levy 1993). Gomer et al. showed that the increased expression of hsp70 heat shock protein family in temperature resistant RIF-1 derivative cell lines did not lead to cross resistance to PDT (Gomer et al. 1990). This suggested that although heat shock proteins are upregulated by PDT treatment, they do not play a significant role in modulating PDT sensitivity. Furthermore it has been suggested by Christensen et al. that hyperthermia

induced inhibition of the repair of photodynamic damage may be responsible for the enhanced cellular destruction seen in combinational therapy (Christensen et al. 1984).

F.2. PDT and Ionizing Radiation

Ionizing radiation damages cellular DNA (Hall 1994). In the case of x-rays, it is estimated that 2/3 of DNA damage in mammalian cells occurs through the indirect action of radiation (Hall 1994). In the indirect action, a secondary electron resulting from absorption of an x-ray photon, interacts with water for example, to produce a hydroxyl radical. The hydoxyl radical then produces damage to DNA by causing a single strand break. Double strand breaks are formed when two single strand breaks are opposite one another or separated by only a few base pairs (Hall 1994).

X-rays are commonly used in cancer therapy. However, in the x-ray treatment of some tumors, it is difficult to avoid destruction of surrounding normal cells. As previously discussed, one of the characteristics of photosensitizers used in PDT is the selectivity with which they accumulate in tumor cells. Therefore, combined PDT and x-ray treatment may allow increased localized therapy of tumors. Also, high concentrations of photosensitizers have often demonstrated direct action on tumor cells without the need for molecular oxygen (Kostron et al. 1988). This feature provides an advantage over conventional ionizing radiation therapy since some areas of a tumor are hypoxic and therefore escape radiation treatment effects (Kostron et al. 1988). For these reasons, researchers have investigated a combination of PDT with ionizing radiation on a variety of tumor cell types (Ben Hur et al. 1988, Biel et al. 1993, Bellnier

and Dougherty 1986, Kavarnos et al. 1994, Kostron et al. 1988). Results investigating PDT and radiation combinational therapy have been conflicting. Concurrent delivery of the two modalities as well as PDT following radiation after various times have resulted in synergistic reduction in survival for some mammalian cell lines but not all mammalian cells (Karvarnos et al. 1994, Kostron 1988, Ben-Hur et al. 1988). It has been suggested that these conflicting results may be due to cell line differences in the sensitivities to PDT induced inhibition of DNA repair caused by radiation damage (Prinze et al. 1992).

F.3. PDT Photosensitizers and Radiosensitization

Recent reports indicate that hematoporphyrin derivative (Kostron et al. 1988), metalloporhyrins (O'Hara et al. 1989) and 5-ALA induced Protoporphyrin IX (Luksiene et al. 1995) can act as radiosensitizers in the absence of light under aerobic conditions. It is suggested that radiosensitization is dependent on the dose of the photosensitizer, the type of sensitizer, and the dose rate at which radiation is administered (Berg et al. 1995). Radiosensitization by photosensitizers is of obvious advantage for the clinical treatment of cancer. In some circumstances where light can not be delivered even by fibre optics, the potentiation of tumor cell kill by sensitizer following ionizing radiation may help to cure patients.

G. The Proposed Study

Previous investigators have characterized differences in sensitivity between RIF-1 and RIF-8A cells following Ph-PDT by clonogenic survival (Singh et al. 1991) and viral capacity (Di Prospero et al. 1996). RIF-8A cells were shown to have morphological and biochemical differences compared to RIF-1 parental cells (Sharkey et al. 1992). Mitochondrial differences between RIF-1 and RIF-8A cells have been suggested to be responsible for the differences in sensitivities to Ph-PDT seen in these cells (Sharkey et al. 1993). The RIF-8A cells were shown to have a cross resistance to cisplatin (Moorehead et al. 1994), but did not show classical multidrug resistance (Singh et al. 1991). This investigation was performed in order to examine the sensitivity of RIF-1 and RIF-8A cells to several different damaging agents including PDT mediated by the novel Ruthenium phthalocyanine photosensitizer JM2929, UV, gamma-radiation, and hyperthermia. Gamma-radiosensitization of RIF-1 and RIF-8A cells by Photofrin or JM2929 was also examined in the present work. The sensitivity of Ph-PDT resistant RIF-P16CL8 and CPR-C1 cells isolated by Luna and Gomer (1991) was also examined following gamma radiation.

It has been previously demonstrated that RIF-8A cells are cross resistant to UV and have an increased capacity to recover from PDT damage compared to RIF-1 parental cells (Di Prospero et al. 1996). It is possible that the cross resistance to UV results from an enhanced capacity for the repair of UV-induced DNA damage in RIF-8A cells. In this research a recombinant non-replicating human adenovirus type 5,

AdCA35*lacZ*, expressing the *lacZ* gene, was used to examine the repair of a UV-damaged reporter gene in RIF-1 and RIF-8A cells. Repair of the UV-damaged reporter gene was examined in untreated cells as well as cells treated with Ph-PDT, JM2929-PDT, UV, cisplatin, and hyperthermia.

CHAPTER 2

PRELIMINARY PDT AND PDT-HYPERTHERMIA COMBINATIONAL EXPERIMENTS USING THE HOECHST FLUOROCHROME ASSAY

ABSTRACT

The Hoechst fluorochrome assay was used in order to study the survival of several radiation induced fibrosarcoma (RIF) cell strains following JM2929 and Photofrin mediated Photodynamic therapy. The DNA staining Hoechst assay served as an alternative method to the more commonly used colony forming assay to conduct dose response studies in the RIF-1 parent cell line, and its Photofrin mediated PDT (Ph-PDT) resistant variants RIF-8A, RIF-P16CL8, and CPR-C1 cells. It was of interest to compare the survival of these cells following PDT damage incurred by a novel photosensitizer, Ruthenium(II) phthalocyanine-bis-(triphenylphosphine-*m*-monosulphonate) potassium salt, or JM2929 (JM2929-PDT). Results indicate a cross resistance to JM2929-PDT of CPR-C1 and RIF-P16CL8 cells, but not of RIF-8A cells. RIF-1 and RIF-8A cells appeared to have similar survival following JM2929-PDT. RIF-1 and RIF-8A cells were also treated with Ph-PDT or JM2929-PDT followed by hyperthermia. JM2929-PDT followed by hyperthermia treatment had no effect on survival of RIF-1 and RIF-8A cells. Results of Ph-PDT followed by hyperthermia in RIF-1 and RIF-8A cells suggest a synergistic effect on survival of RIF-8A cells but not RIF-1 cells. This result may suggest hyperthermia induced inhibition of Ph-PDT repair at lower levels of Ph-PDT induced cellular damage, in Ph-PDT resistant cells.

INTRODUCTION

Photodynamic Therapy (PDT) is a novel method of cancer treatment which uses the preferential activation of photosensitizer molecules accumulated in tumor cells to cause cellular damage (Pass 1993, Manyak et al. 1988, Dougherty and Marcus 1992, Wieman and Fingar 1992). The exact mechanism(s) involved in PDT induced cellular destruction is still unclear, however Phase III clinical trials in PDT are already underway (Dougherty and Marcus 1992). Photofrin, a haematoporphyrin derivative photosensitizer activated at 620nm, has been used in these clinical trials but the search continues for more efficient sensitizers that are excited at longer wavelengths for increased tissue penetration, and have less side effects such as prolonged light sensitivity in patients.

The Ruthenium phthalocyanine (JM2929) is a novel photosensitizer that is non-toxic and is activated at 650nm. JM2929 is water soluble, and has a high quantum yield (Singh personal communication). Phthalocyanines which differ in their metal ion and degree of sulphonation have been shown to accumulate in, and cause damage to, subcellular targets such as plasma membranes, mitochondrial membranes, lysosomes, as well as nuclear DNA (Evans et al. 1989, Ben Hur et al. 1987, Ben Hur et al. 1991, Moan et al. 1992). The specific targets for JM2929 have not yet been determined.

The radiation induced fibrosarcoma (RIF-1) tumor system was developed by Twentyman et al. (1980) and has served as an in vivo/in vitro model for PDT studies (Twentyman et al. 1980). In order to develop a better understanding of the

mechanism(s) involved in PDT induced cytotoxicity, PDT resistant cells have been developed and characterized for cellular changes that may be responsible for the resistant phenotype. The Ph-PDT resistant RIF-8A variant has been derived from RIF-1 cells (Singh et al. 1991). These Ph-PDT resistant cells were shown to have altered mitochondrial morphology and function (Sharkey et al. 1992, Sharkey et al. 1993). The RIF-8A cells have an increased number of smaller and denser mitochondria, as well as increased levels of ATP production compared to the RIF-1 parental cells (Sharkey et al. 1993). It has been suggested that these differences contribute to the Ph-PDT resistance observed in the RIF-8A cells (Sharkey et al. 1993).

RIF-1 and RIF-1G cells have been identified with different names but are both parental cell lines belonging to the original RIF tumor developed by Twentyman et al. (1980). RIF-P16CL4 and RIF-P16CL8 cells derived from RIF-1G parent cells also have increased resistance to Ph-PDT (Luna and Gomer 1991). RIF-P16CL4 and RIF-P16CL8 cells were both shown to have a small increase in resistance to an ionizing radiation dose of 15 Gy (but not to doses in the range of 0-10 Gy) compared to RIF-1G parent cells (Luna and Gomer 1991). RIF-P16CL4 cells were also shown to have an increased sensitivity to hyperthermia. CPR-C1 cells derived from RIF-P16CL8 cells have increased resistance to Ph-PDT compared to RIF-P16CL8 cells and RIF-1G parent cells (Singh unpublished observation). In the present work, the survival of RIF-1, RIF-8A, RIF-1G, RIF-P16CL8 and CPR-C1 cells following JM2929-PDT was examined using the Hoechst Fluorochrome assay.

Increased cell kill following the combination PDT and hyperthermia, compared to cell kill resulting from each treatment alone, is referred to as synergy. When no increase in cell kill results, the effects of combined PDT and hyperthermia treatments are referred to as being additive. The effects of PDT in combination with hyperthermia have been investigated by many researchers using different tumor cells and different photosensitizers (Mang 1990, Glassberg et al. 1991, Berns et al. 1984, Waldow et al. 1987). Results suggest a synergistic effect on the reduction of cell survival when PDT is followed by hyperthermia treatment (Waldow et al. 1985, Christensen et al. 1984). In vivo, a synergistic effect following Ph-PDT and hyperthermia has been observed on the RIF-1 tumor (Henderson et al. 1985). Henderson et al. (1985) suggested that for RIF-1 tumor cells, Ph-PDT and hyperthermia treatments induced similar cellular damage but that the mechanism(s) of damage were different for these two modalities (Henderson et al. 1985). The effects of hyperthermia treatment following either JM2929-PDT or Ph-PDT were examined for RIF-8A and RIF-1 cells in vitro.

The clonogenic assay can be used to measure the reproductive integrity of individual cells following treatment with a damaging agent (Hall 1994). The Hoechst fluorochrome assay can be used to measure the amount of DNA that is present following the treatment of cells with a damaging agent. The Hoechst fluorochrome assay requires the exposure of nuclear DNA to a fluorochrome which, when bound to DNA, is excited at 360 nm to emit at 460 nm. Fluorochrome which is not bound to DNA provides background fluorescence. The following work used the Hoechst Fluorescence assay to characterize the sensitivity of RIF-1 and RIF-1 derived variant

cells to JM2929-PDT, Ph-PDT, and hyperthermia treatment following either Ph-PDT or JM2929-PDT.

MATERIALS AND METHODS

Cells

RIF-1 cells were provided by Dr. B. Henderson, Roswell Park Memorial Institute, Buffalo, New York. RIF-8A cells were obtained from Dr. Gurmit Singh, Hamilton Regional Cancer Clinic and McMaster University, Dept. of Pathology, Hamilton, ON. RIF-1G, RIF-P16CL8 and CPR-C1 were obtained from Dr. C. Gomer at the Clayton Ocular Oncology Centre, Children's Hospital of Los Angeles, Los Angeles, CA. All cell lines were grown in monolayer and maintained in Eagles α-minimal essential medium α-MEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (100 μg/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B; Gibco BRL Mississauga ON) (growth media), and incubated at 37°C (5% CO_2/95% air in a humid environment).

Photosensitizers and Light Source

Photofrin was obtained from Quadralogic Technologies Inc. (Vancouver, B.C.). JM2929 was obtained from Johnson Matthey Inc. (PA, USA). Both sensitizers were diluted to concentrations between 5-20 mg/mL in growth media. Cells were pretreated with photosensitizers for 18 hours prior to red light exposure on a 100cm X 50cm light diffusing surface illuminated by a bank of fluorescent tubes (Philips type TL/83), filtered with red acetate filters (Roscolux, No.19, Rosco, CA) to give wide band illumination above 585 nm.

The energy fluence rate was approximately 9.2 $J/m^2/sec$. Exposure for 5 minutes resulted in an incident energy fluence of approximately 2 760 J/m^2.

Photofrin or JM2929 mediated PDT

$2x10^4$ cells in 1mL of growth media were seeded in 24 well plastic culture plates (Falcon, Lincoln Park, NJ) and allowed to adhere for 6 hours. Cells were treated with photosensitizer and incubated for 18 hours. Growth media containing photosensitizer was then aspirated and replaced with fresh prewarmed growth media immediately prior to 5 minutes of illumination with red light. Cells were then incubated at 37°C (5% CO_2/95% air in a humid environment) for an appropriate amount of time prior to measurement of cell number by the Hoechst Fluorochrome Assay. All work involving photosensitizer was conducted in minimal ambient light conditions.

PDT and Hyperthermia Combinational Treatments

Cells were seeded and exposed to PDT as described above. Immediately following light exposure, plates were sealed using water resistant PVC tape and placed into a water bath prewarmed to 43°C ± 0.25°C. Plates were completely submerged in water for one hour in minimal ambient light conditions.

Hoechst Fluorochrome Assay

Following the appropriate incubation period, media from each well was aspirated and 1.5 mL phosphate buffer saline (PBS) (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4

and 1.75 mM KH_2PO_4) was added to rinse each well. PBS was then aspirated and 1.0 mL sterile H_2O added to each well. Plates were incubated for 24 hours at 37°C (5% C_{O2}/95% air in a humid environment) to allow cell lysis to occur. Plates were then placed in a -20°C freezer and thawed to ensure complete cell lysis. The DNA staining Hoechst dye was diluted in TNE buffer (10mM Tris base, 1mM EDTA, and 2M NaCl) for a final concentration of 0.025 mg/mL. 1mL of Hoechst TNE solution was added to each well and DNA fluorescence was measured using the Cytofluor 2350 manufactured by Millipore.

Colony Forming Assay

All colony forming assays were performed in 6 well plastic petri dishes. Appropriate numbers of cells were seeded in order to count between 100-200 colonies per well post treatment. Plates were incubated at 37°C (5% CO_2/95% air in a humid environment) for 4 days prior to staining with methylene blue (5% methylene w:v in 70% methanol and 30% dH_20). Only colonies greater than 20 cells were counted. In each experiment, colony counts for each treatment to cells were the average of triplicate determinations.

Standard Curves of RIF Cells

0 - 2 x 10^5 cells were seeded into 24-well plastic culture plates in growth media. Cells were allowed to adhere for 4 hours prior to measurement of DNA fluorescence by the Hoechst technique described above.

RESULTS

Standard Curves of RIF Cells

In the Hoechst technique, the amount of DNA fluorescence is used as a measure of cell number. In order to confirm that the number of cells present in a well correlated with the amount of DNA fluorescence, standard curves were obtained for all RIF cells. Results shown in Fig.1 indicate that for each cell line data was consistent with a linear relationship between the number of cells present and the DNA fluorescence count obtained from cytofluor readings.

Growth Curves for RIF cells

To determine appropriate incubation times for untreated control cells from each cell line to go through a minimum of one doubling time prior to measurement of DNA fluorescence by Hoechst staining, growth curves were conducted. Results show that a 72 hour incubation period was sufficient for the fluorescence count to increase by at least a factor of 2 in CPR-C1, RIF-1G, RIF-1 and RIF-8A cells (Figure 2). RIF-P16CL8 cells however required a minimum of 120 hours for the DNA fluorescence count to double.

Sensitivity of RIF-1G, RIF-P16CL8, and CPR-C1 cells to JM2929-PDT

Cells were exposed to varying doses (5-20μg/mL) of JM2929 and approximately 2.76×10^3 J/m^2 incident energy fluence. Survival for RIF-1G, RIF-P16CL8, and CPR-C1 cells were determined using the Hoechst Fluorochrome assay in three independent

experiments. No dark toxicity at 20 μg/mL drug dose was observed. No effect on survival was observed following 2.76 x 10^3 J/m^2 light dose alone. Results from a typical experiment shown in Figure 3 suggest that the relative sensitivity to JM2929-PDT of Ph-PDT resistant CPR-C1 cells, intermediate Ph-PDT resistant RIF-P16CL8 cells, and Ph-PDT sensitive RIF-1G parent cells, were similar to previously examined relative sensitivities of these cells to Ph-PDT (Luna and Gomer 1991, Singh unpublished observation).

Ph-PDT and JM2929-PDT sensitivity in RIF-1 and RIF-8A cells

RIF-1 and RIF-8A cells were exposed to either Photofrin or JM2929 doses ranging from 5-20μg/mL and approximately 2.76 x 10^3 J/m^2 incident energy fluence. Survival was determined by the Hoechst Fluorochrome assay in three independent experiments conducted in triplicate. No dark toxicity at 20 μg/mL drug dose was observed. No effect on survival was observed following 2.76 x 10^3 J/m^2 light dose alone. Figure 4 shows results from a typical experiment. Ph-PDT results are consistent with previously published clonogenic survival results that indicate a high level of resistance to Ph-PDT in RIF-8A cells compared to RIF-1 cells (Figure 4A) (Singh et al. 1991). However, survival of RIF-1 and RIF-8A cells as measured by Hoechst Fluorochome assay was similar over the range of JM2929-PDT doses examined.

PDT and Hyperthermia Combinational Treatments

RIF-1 and RIF-8A cells were treated with either Ph-PDT or JM2929-PDT and then further exposed to 1 hour hyperthermia treatment at 43°C ± 0.25. Survival

following each combined treatment of either RIF-1 or RIF-8A cells was measured by the Hoechst fluorochrome assay as well as the colony forming assay. Each assay was conducted once in triplicate trials. Survival of RIF-1 cells as measured by Hoechst fluorochrome assay and the colony forming assay following combined Ph-PDT and hyperthermia treatment are shown in Figure 5 and 6 respectively. Results indicate no synergy between Ph-PDT and hyperthermia over the range of doses and heat treatments examined. However synergistic effects on survival were seen for RIF-8A cells by the Hoechst fluorochrome assay (Figure 7) and colony forming assay (Figure 8) following Ph-PDT and hyperthermia combinational treatment.

In the case of JM2929-PDT and hyperthermia combinational treatment, no synergistic effect on survival was detected in RIF-1 or RIF-8A cells over the range of doses and heat treatments examined by either the Hoechst fluorochrome assay or the colony forming assay (Figs. 9,10,11,12).

Figure 1: Standard Curves of DNA Fluorescence vs. Number of Cells for RIF Cells

Standard curves for CPR-C1 (-■-), RIF-1G (-▲-), RIF-P16CL8 (-◆-), RIF-1(-▼-), and RIF-8A (-●-) cells were obtained by the Hoechst fluorochrome technique. Shows results of a single experiment conducted in triplicate trials. Each data point is the arithmetic mean ± standard error.

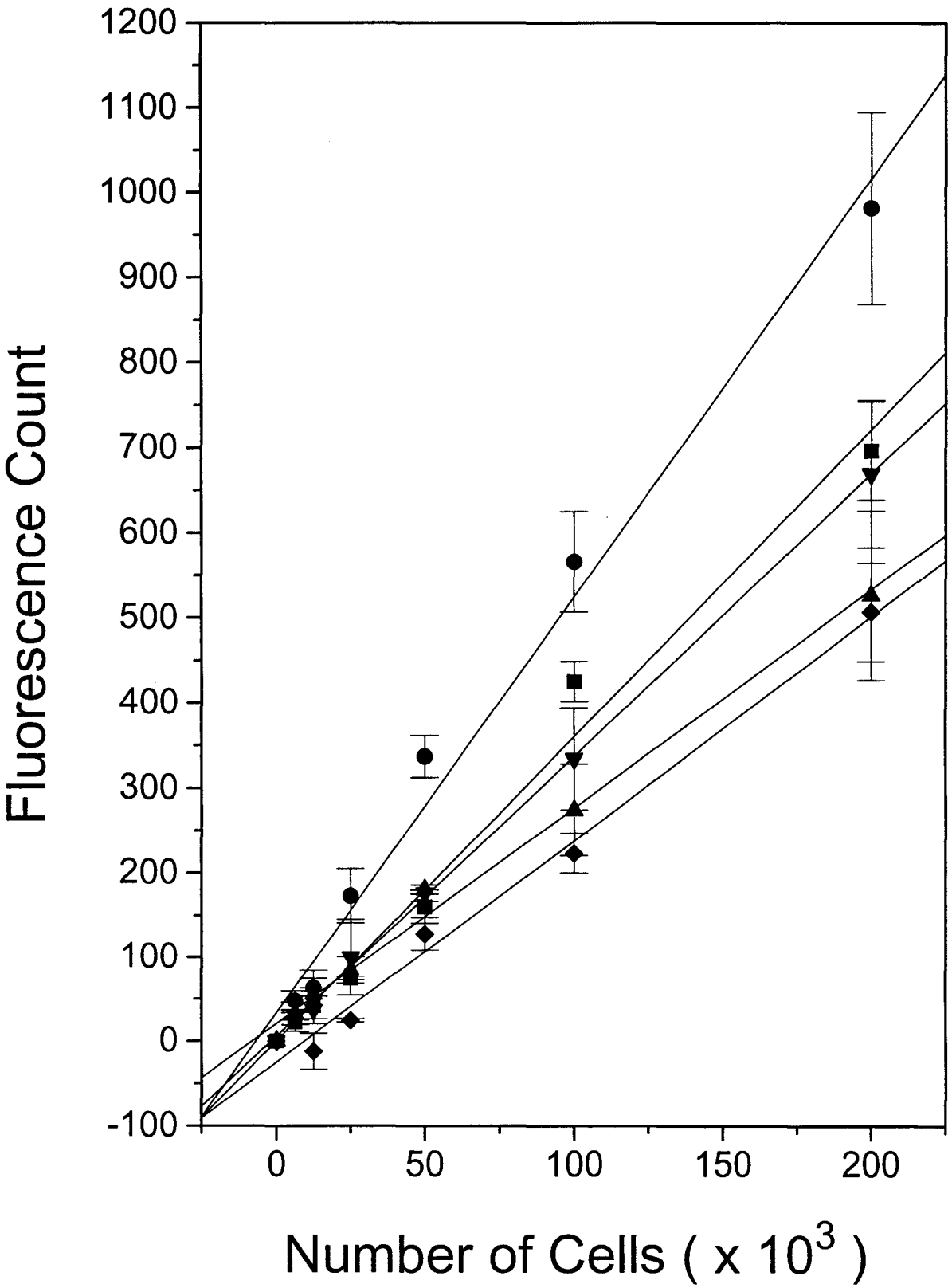

Fluorescence Count
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
0
50
100
150
200
Number of Cells (x 10^3)

Figure 2: Growth Curves of RIF cells

Growth curves for RIF-1 (-■-), RIF-8A (-●-), RIF-1G (-▲-), RIF-P16CL8 (-▼-) and CPR-C1 (-◆-) cells were generated by plotting time of incubation (prior to the Hoechst DNA fluorescence measurement) vs. DNA fluorescence count. Shows results of a single experiment conducted in triplicate. Each data point is the arithmetic mean ± standard error.

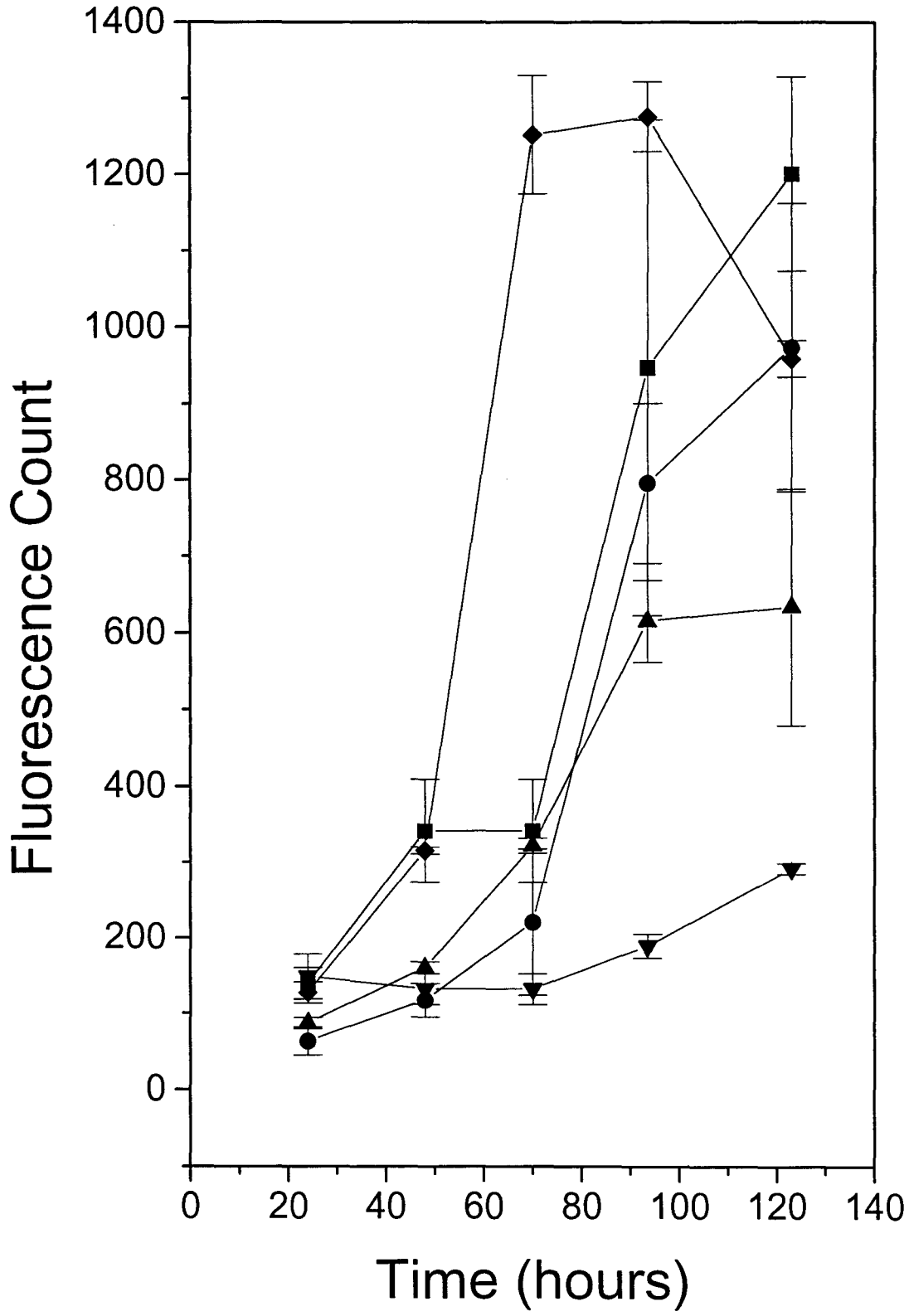

Fluorescence Count
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
Time (hours)

Figure 3: Survival of RIF-1G, RIF-P16CL8, and CPR-C1 cells following JM2929-PDT.

RIF-1G (-■-), RIF-P16CL8 (-●-), and CPR-C1 (-◆-) cells were exposed to varying doses of JM2929-PDT and their survival determined using the Hoechst Fluorochrome assay. Shows results from a typical experiment. Each data point represents the arithmetic mean ± standard error. Similar results were obtained in three independent experiments.

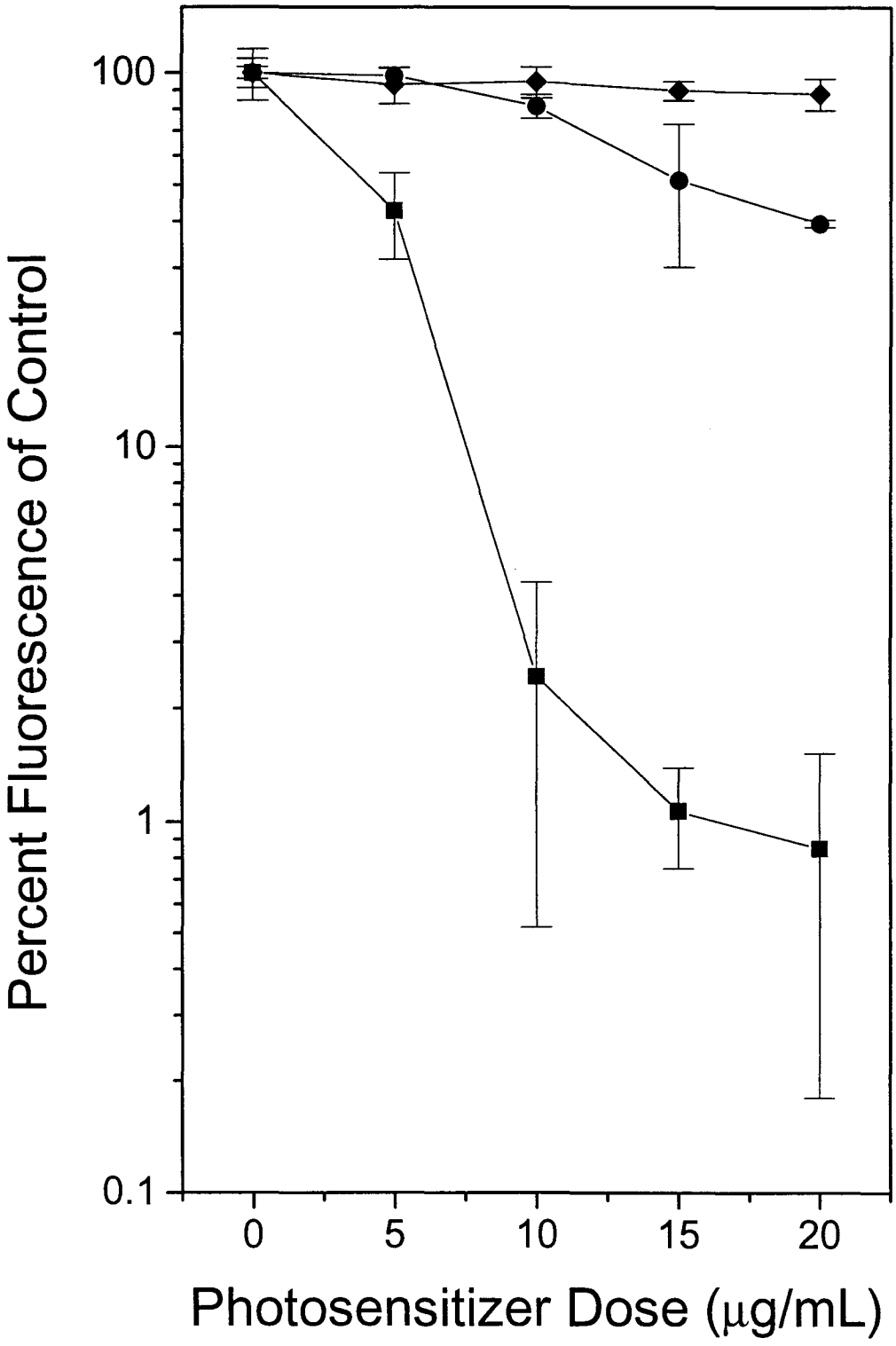

100
10
1
0.1
Percent Fluorescence of Control
0
5
10
15
20
Photosensitizer Dose (μg/mL)

Figure 4: Survival of RIF-1 and RIF-8A cells following exposure to JM2929-PDT or Ph-PDT

RIF-1 (-■-) and RIF-8A (-●-) cells were exposed to varying doses of (A) Ph-PDT or (B) JM2929-PDT and their sensitivity determined using the Hoechst Fluorochrome assay. Shows results from a typical experiment. Each data point represents the arithmetic mean ± standard error. Similar results were obtained in three independent experiments.

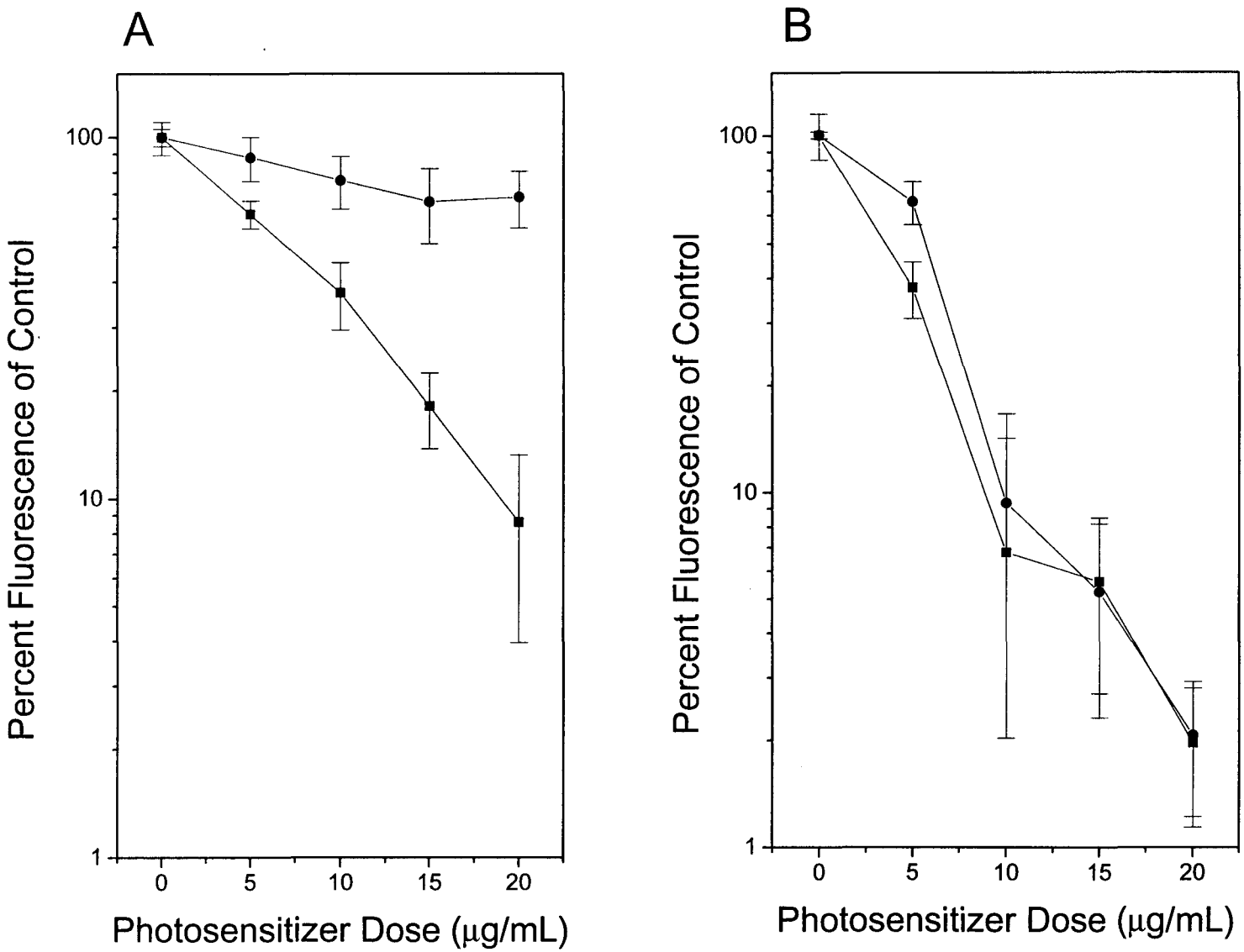

A
B
100
10
1
Percent Fluorescence of Control
0
5
10
15
20
Photosensitizer Dose (µg/mL)

Figure 5: Survival of RIF-1 cells following Ph-PDT and Hyperthermia treatment measured by Hoechst Fluorochrome assay.

RIF-1 cells were exposed to Ph-PDT and hyperthermia treatment. Survival for Ph-PDT alone (PDT), hyperthermia alone (HS) and PDT combined with hyperthermia (PDT + HS) in RIF-1 cells was determined using the Hoechst Fluorochrome assay. Results are from a single experiment conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

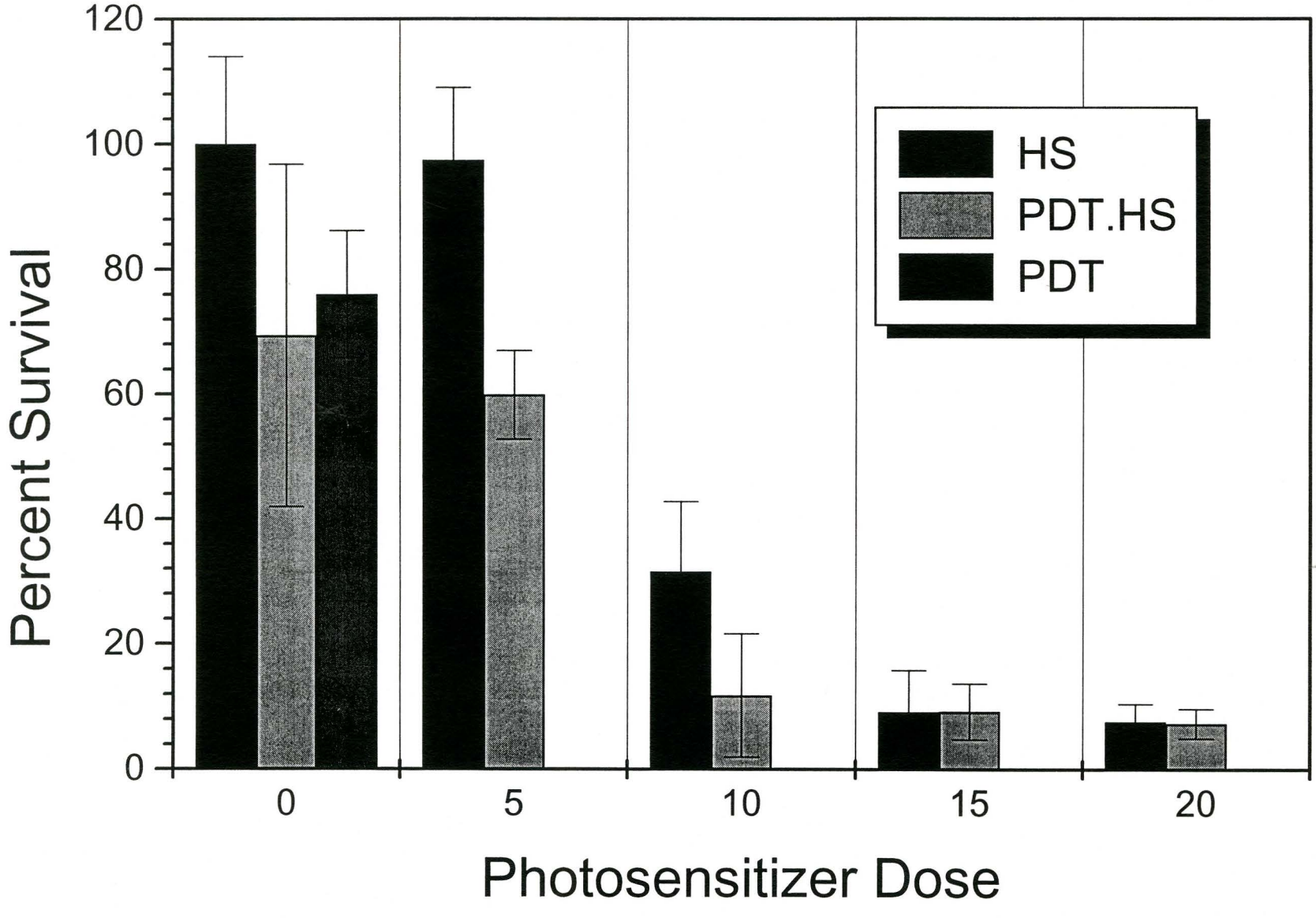

120
100
80
60
40
20
0
Percent Survival
0
5
10
15
20
Photosensitizer Dose
HS
PDT.HS
PDT

Figure 6: Survival of RIF-1 cells following Ph-PDT and Hyperthermia treatment measured by colony forming assay.

RIF-1 cells were exposed to Ph-PDT and hyperthermia treatment. Survival for Ph-PDT alone (PDT), hyperthermia alone (HS) and PDT combined with hyperthermia (PDT + HS) in RIF-1 cells was determined using the colony forming assay. Results are from a single experiment conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

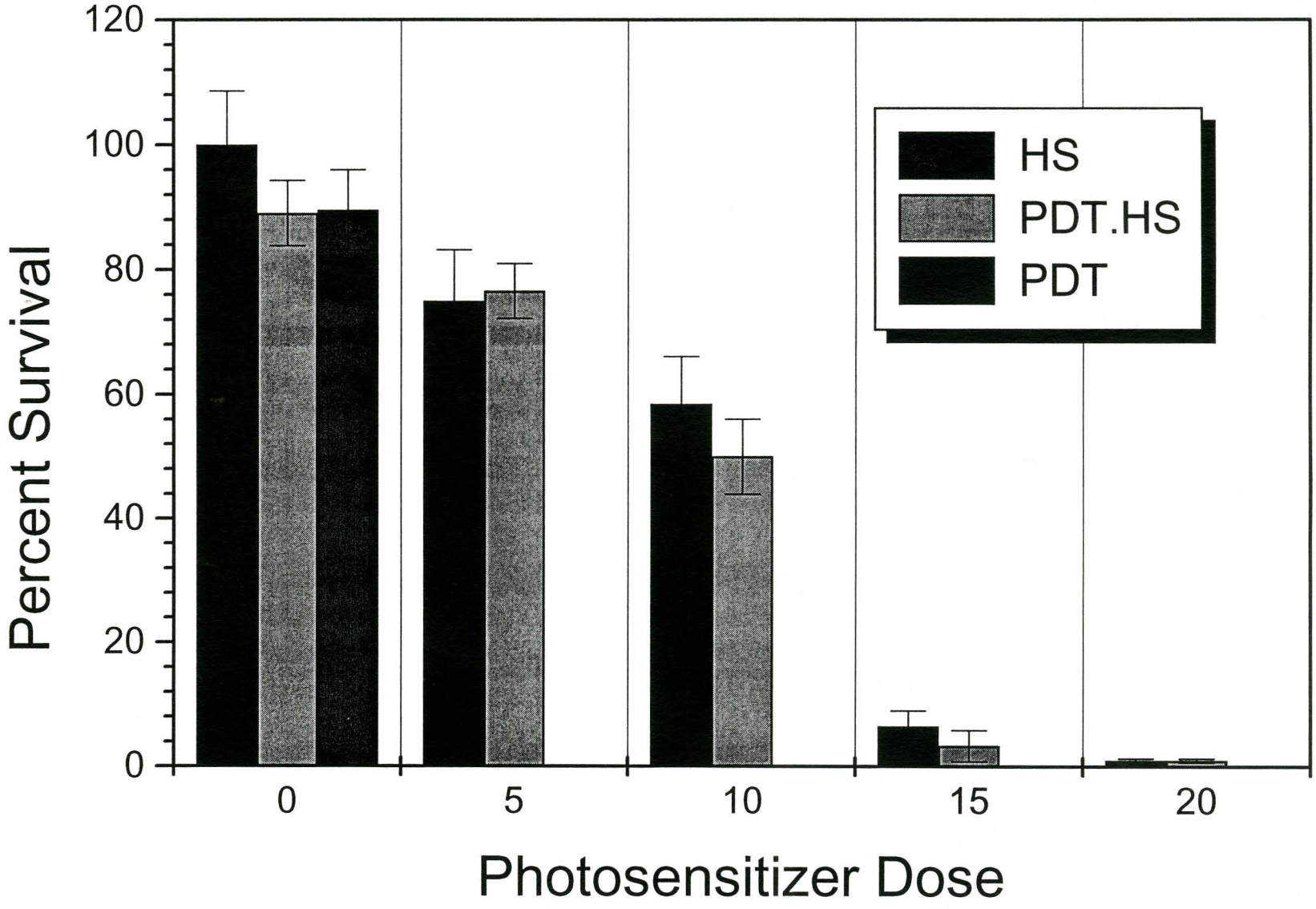

120
100
80
60
40
20
0
Percent Survival
0
5
10
15
20
Photosensitizer Dose
HS
PDT.HS
PDT

Figure 7: Survival of RIF-8A cells following Ph-PDT and Hyperthermia treatment measured by Hoechst Fluorochrome assay.

RIF-8A cells were exposed to Ph-PDT and hyperthermia treatment. Survival for Ph-PDT alone (PDT), hyperthermia alone (HS) and PDT combined with hyperthermia (PDT + HS) in RIF-8A cells was determined by Hoechst Fluorochrome assay. Results are from a single experiment conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

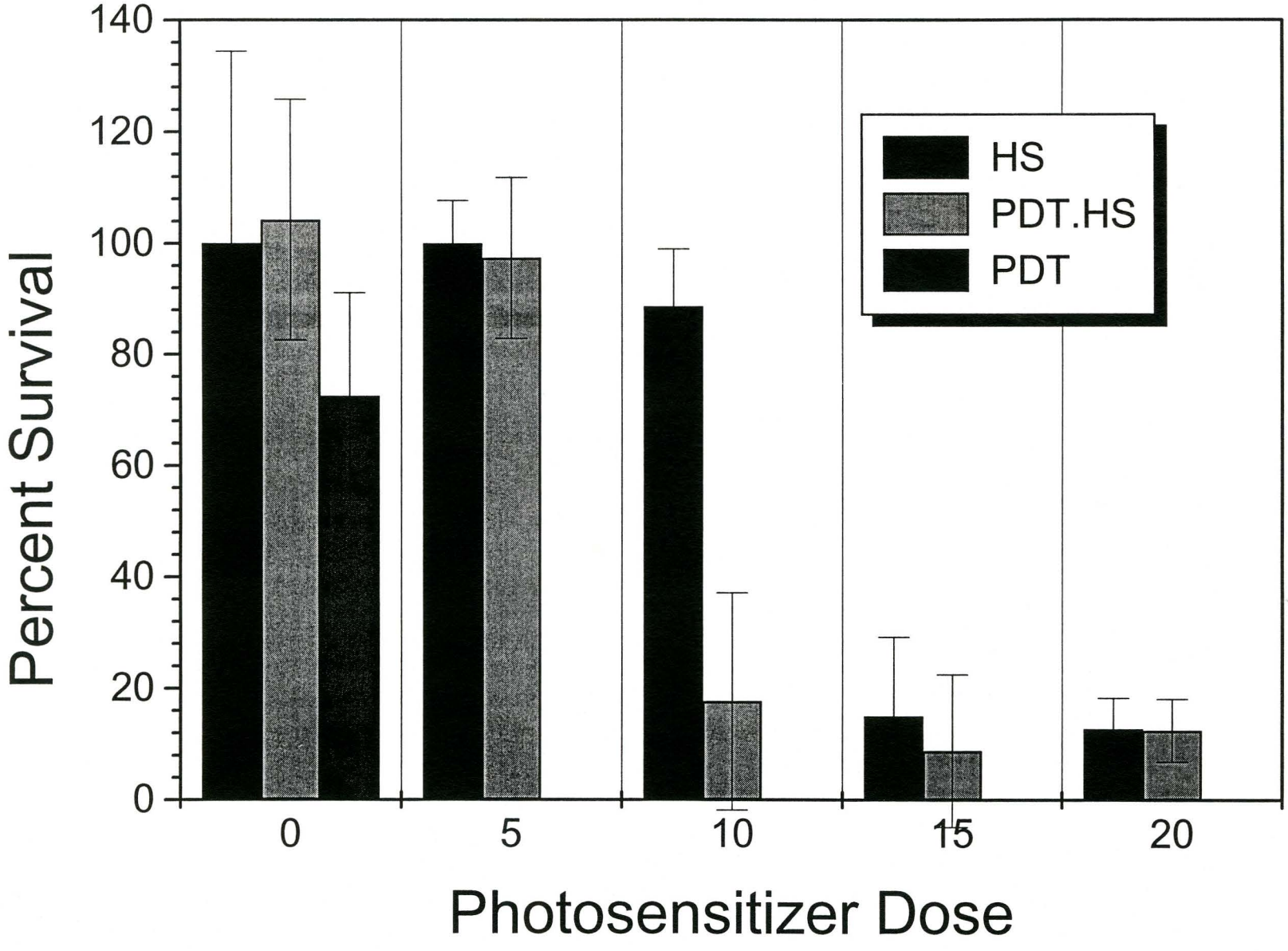

140
120
100
80
60
40
20
0
Percent Survival
HS
PDT.HS
PDT
0
5
10
15
20
Photosensitizer Dose

Figure 8: Survival of RIF-8A cells following Ph-PDT and Hyperthermia treatment measured by colony forming assay.

RIF-8A cells were exposed to Ph-PDT and hyperthermia treatment. Survival for Ph-PDT alone (PDT), hyperthermia alone (HS) and PDT combined with hyperthermia (PDT + HS) in RIF-8A cells was determined using the colony forming assay. Results are from a single experiment conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

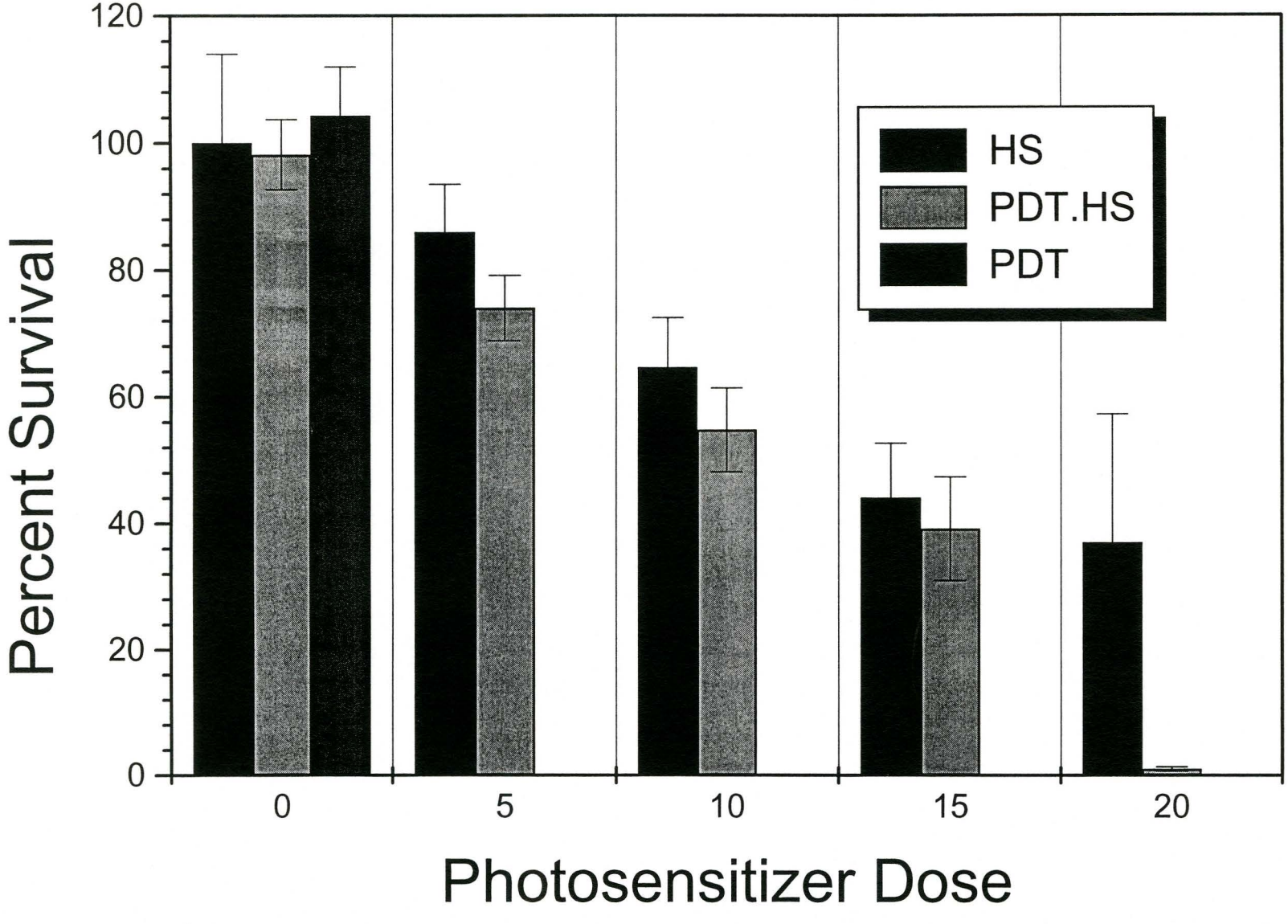

120
100
80
60
40
20
0
Percent Survival
HS
PDT.HS
PDT
0
5
10
15
20
Photosensitizer Dose

Figure 9: Survival of RIF-1 cells following JM2929-PDT and Hyperthermia treatment measured by Hoechst Fluorochrome assay.

RIF-1 cells were exposed to JM2929-PDT and hyperthermia treatment. Survival for JM2929-PDT alone (PDT), hyperthermia alone (HS) and PDT combined with hyperthermia (PDT + HS) in RIF-1 cells was determined by Hoechst Fluorochrome assay. Results are from a single experiment conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

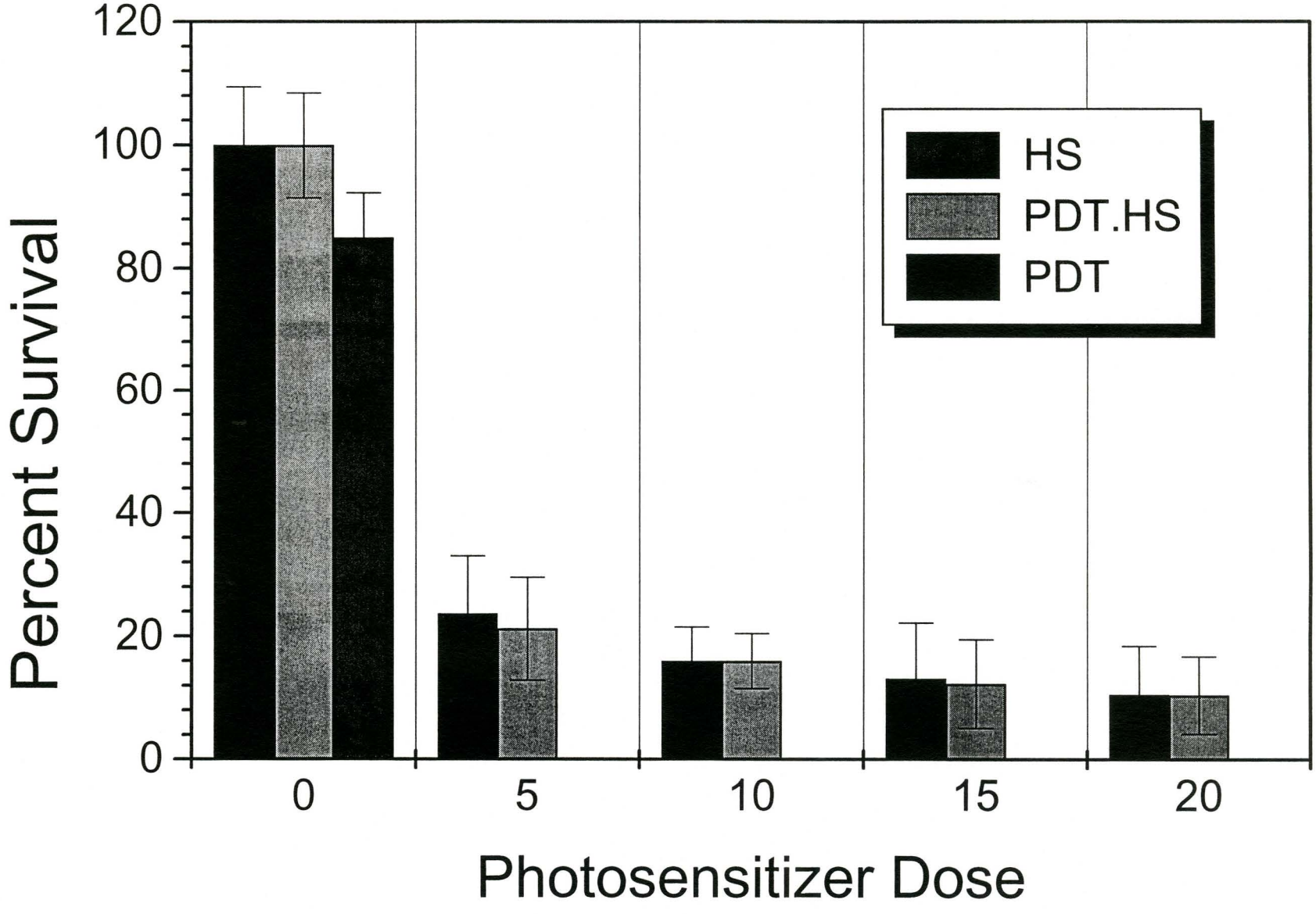

120
100
80
60
40
20
0
Percent Survival
HS
PDT.HS
PDT
0
5
10
15
20
Photosensitizer Dose

Figure 10: Survival of RIF-1 cells following JM2929-PDT and Hyperthermia treatment measured by colony forming assay.

RIF-1 cells were exposed to JM2929-PDT and hyperthermia treatment. Survival for JM2929-PDT alone (PDT), hyperthermia alone (HS) and PDT combined with hyperthermia (PDT + HS) in RIF-1 cells was determine using colony forming assay. Results are from a single experiment conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

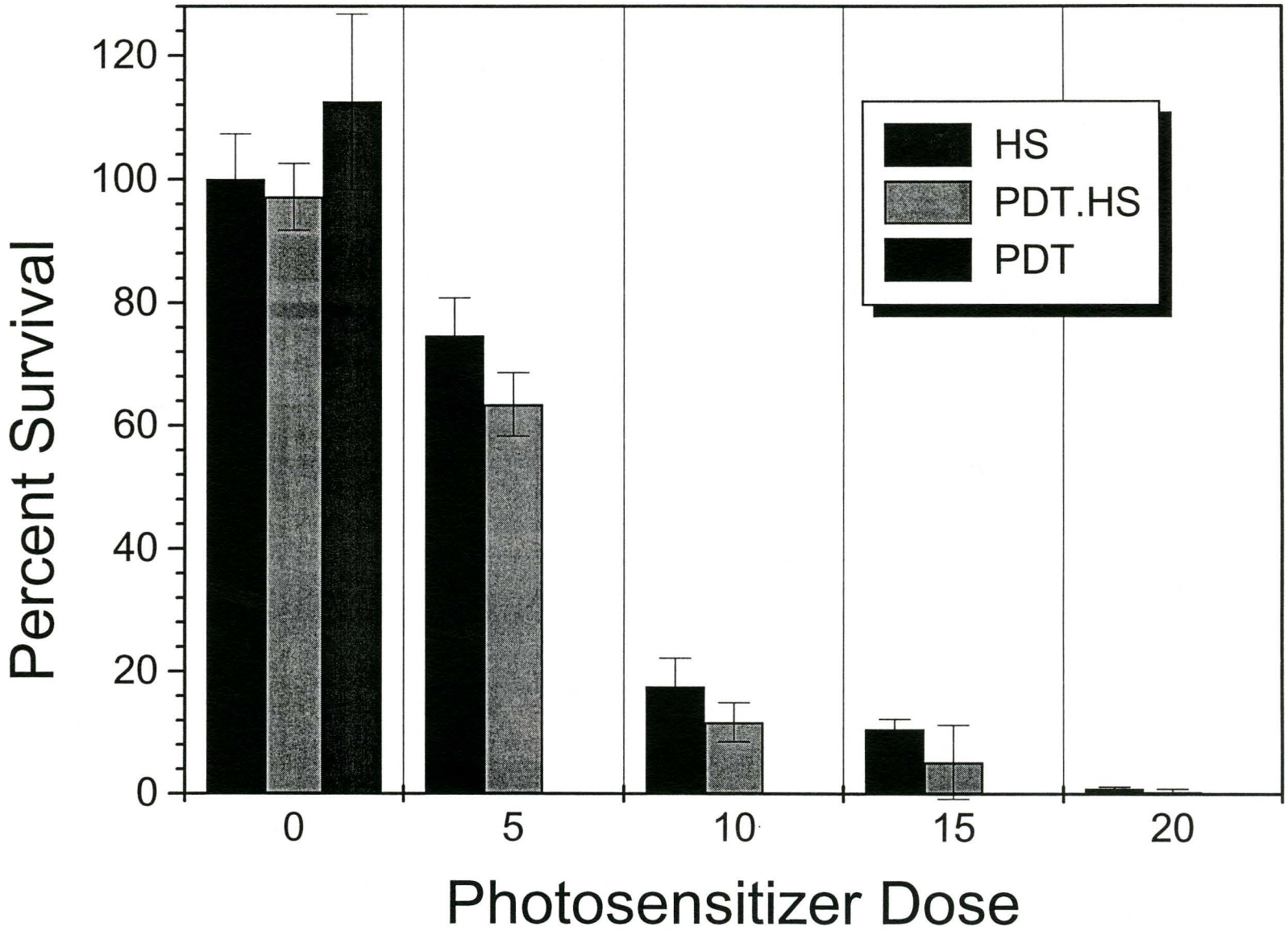

Percent Survival
120
100
80
60
40
20
0
0
5
10
15
20
Photosensitizer Dose
HS
PDT.HS
PDT

Figure 11: Survival of RIF-8A cells following JM2929-PDT and Hyperthermia treatment measured by Hoechst Fluorochrome assay.

RIF-8A cells were exposed to JM2929-PDT and hyperthermia treatment. Survival for JM2929-PDT alone (PDT), hyperthermia alone (HS) and PDT combined with hyperthermia (PDT + HS) in RIF-8A cells was determined using the Hoechst Fluorochrome assay. Results are from a single experiment conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

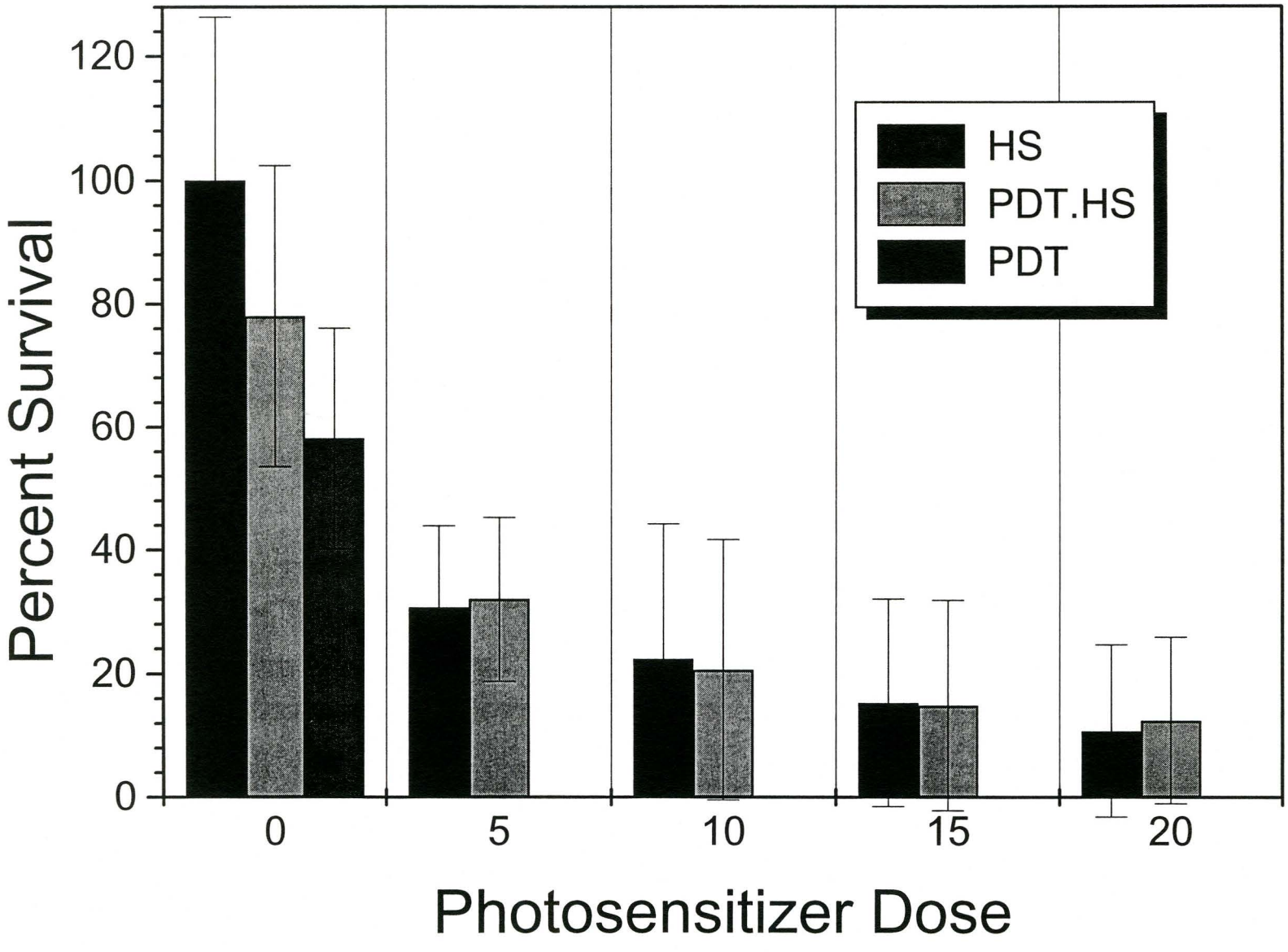
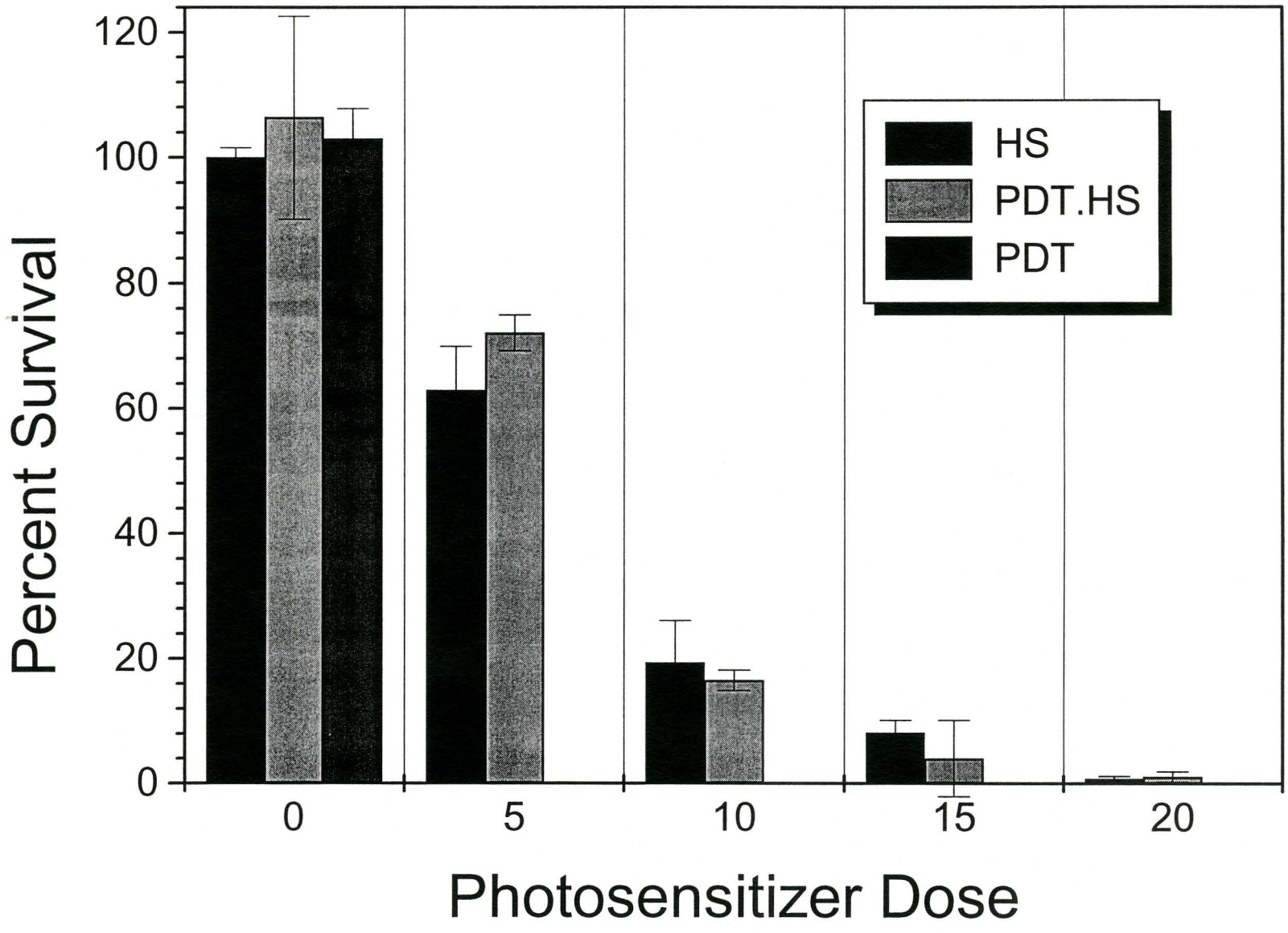

Percent Survival
120
100
80
60
40
20
0
HS
PDT.HS
PDT
0
5
10
15
20
Photosensitizer Dose

Figure 12: Survival of RIF-8A cells following JM2929-PDT and Hyperthermia treatment measured by colony forming assay.

RIF-8A cells were exposed to JM2929-PDT and hyperthermia treatment. Survival for JM2929-PDT alone (PDT), hyperthermia alone (HS) and PDT combined with hyperthermia (PDT + HS) in RIF-8A cells was determined using the colony forming assay. Results are from a single experiment conducted in triplicate. Each bar represents the arithmetic mean ± standard error.


Percent Survival
120
100
80
60
40
20
0
0
5
10
15
20
Photosensitizer Dose
HS
PDT.HS
PDT

DISCUSSION

By colony forming assay, the RIF-8A cells have been shown to have an increased survival compared to the RIF-1 parental cells at the light dose of 2.7 x 10^3 J/m^2 and 20 μg/mL Photofrin (Singh et al. 1991). In the present work, results from the Hoechst fluorochrome assay were consistent with clonogenic survival results suggesting increased resistance to Ph-PDT by RIF-8A cells compared to RIF-1 cells. The RIF-1 and RIF-8A cells have differences in their mitochondrial morphology and function (Sharkey et al. 1992). Ph-PDT resistant RIF-8A cells show a cross resistance to cisplatin (Moorehead et al. 1994). Cross resistance to Ph-PDT has also been seen in the cisplatin resistant C13 cell line derived from the human ovarian carcinoma 2008 cell line (Sharkey et al. 1993). Interestingly, C13 and 2008 parent cells contain similar mitochondrial differences to those reported between RIF-8A and RIF-1 parental cells (Sharkey et al. 1993, Moorehead et al. 1994). Porphyrin based photosensitizers such as Photofrin, have been observed to accumulate and cause photoinduced damage in plasma and mitochondrial membranes (Kessel 1977, Moan et al. 1989, Gomer et al. 1988, Hilf et al. 1987). It has been suggested that mitochondrial changes in the RIF-8A cells may be responsible for the Ph-PDT resistant phenotype observed in these cells (Sharkey et al. 1993).

Phthalocyanines have previously been shown to also accumulate in surfaces of the plasma membrane, mitochondria, lysosomes, as well as the nucleus (Evans et al. 1989, Ben Hur et al. 1987, Ben Hur et al. 1991, Moan et al. 1992). The specific targets

of the phthalocyanine JM2929 have not yet been defined. It was therefore of interest to compare the cytotoxicity of JM2929-PDT with that seen for Ph-PDT in RIF cells. Results obtained from the DNA staining Hoechst fluorochrome assay indicate no difference in survival between RIF-1 and RIF-8A cells for the doses of JM2929-PDT delivered. This result suggests that the cellular targets for JM2929-PDT and Ph-PDT induced damage are not identical in RIF-1 and RIF-8A cells and that changes in mitochondrial morphology and function do not result in increased survival for Ph-PDT resistant RIF-8A cells following JM2929-PDT.

Survival of CPR-C1, RIF-P16CL8, and RIF-1G cells to JM2929-PDT were also examined using the Hoechst Fluorochrome assay. By clonogenic survival assay, RIF-P16CL8 cells were shown to have increased resistance to Ph-PDT compared to RIF-1G cells (Luna and Gomer 1991). Results using the Hoechst fluorochrome assay suggest that RIF-P16CL8 cells are cross resistant to JM2929-PDT compared to RIF-1G cells following JM2929-PDT. Using the colony forming assay, CPR-C1 cells have been shown to have increased resistance to Ph-PDT compared to RIF-1G cells (Singh unpublished observation). Ph-PDT resistance was also shown to be higher in CPR-C1 cells compared to RIF-P16CL8 cells (Singh unpublished observation). The Hoechst Fluorochrome assay showed that CPR-C1 cells maintained increased resistance compared to RIF-P16CL8 and RIF-1G cells following JM2929-PDT. The relative sensitivities of CPR-C1, RIF-P16CL8, and RIF-1G cells were similar following Ph-PDT and JM2929-PDT. This result suggests that the mechanism of induced Ph-PDT resistance in RIF-8A cells is different from that in CPR-C1 and RIF-P16CL8 cells.

Earlier PDT studies reported that hyperthermia may increase the response of mouse tumor cells in vivo and in vitro to haematoporphyrin mediated PDT (Waldow et al. 1984, Melloni et al. 1984, Christensen et al. 1984, Waldow et al. 1985, Svaasand 1985, Glassberg et al. 1991). Synergy between PDT and hyperthermia has been correlated with sequence of treatment. Waldow et al. (1985) found that for the SMT-F mammary carcinoma in mice in vivo, the immediate exposure of tumor cells to hyperthermia following PDT treatment was most effective in potentiating effects of PDT and increasing long term tumor control (Waldow et al. 1985). Whereas only additive effects of the independent treatments were found when heat treatment was applied 0-8 hours prior to PDT exposure. Also, synergistic effects of heat followed by PDT appeared to decrease with increasing time between PDT and heat administration (Waldow et al. 1985). Henderson et al. (1985) showed that in RIF-1 tumor cells in vitro, heat treatment alone at 44°C for 30 minutes led to an immediate reduction in the number of clonogenic tumor cells, followed by some additional cell death for 4 hours, and subsequent recovery of clonogenicity. Treatment of RIF-1 cells with heat prior to PDT resulted in survival kinetics similar to heat treatment alone. Synergy was only seen when Ph-PDT was followed by hyperthermia. It was suggested that despite similar microscopic and macroscopic appearances following Ph-PDT or hyperthermia treatments, these two modalities lead to tumor destruction by different mechanisms in RIF tumor cells (Henderson et al. 1985). Gomer et al. (1990) found elevated levels of heat shock proteins (hsp) of molecular weight 70 000 in thermotolerant RIF cells. However, these cells did not show cross resistance to Ph-PDT (Gomer et al. 1990). This

suggested that mechanisms of in vitro cytotoxicity were different for Ph-PDT and hyperthermia even though possible subcellular targets such as the plasma membrane, and types of damage such as protein denaturation, were similar for the two treatments (Gomer et al. 1990). Preliminary results in our laboratory using immunofluorescence studies suggest elevated levels of hsp60 in RIF-8A cells (K.Adams personal communication). The role of increased levels of hsp60 in RIF-8A cells is still undetermined.

Preliminary results of JM2929-PDT or Ph-PDT and hyperthermia treatments obtained using the Hoechst Fluorochrome assay and the colony forming assay suggest synergistic effects on survival of RIF-8A cells following small amounts of Ph-PDT induced cytotoxicity and hyperthermia treatment. Synergy between the two modalities was not observed following increased Ph-PDT induced cytotoxicity in RIF-8A cells. This result may suggest that hyperthermia for one hour at 43°C ± 0.25 interferes with cellular repair processes that are induced by lower levels of Ph-PDT damage in RIF-8A cells. No synergistic effect on survival was seen for RIF-1 cells following the doses of Ph-PDT and hyperthermia examined. In the case of JM2929-PDT and hyperthermia treatment, synergistic effects on survival were not observed over the range of doses and heat treatment examined, for either RIF-1 or RIF-8A cells. These results support the suggestion that damage induced in RIF-1 and RIF-8A cells following Ph-PDT or JM2929-PDT is different. This may be due to different subcellular targets for Ph-PDT and JM2929-PDT induced cytotoxicity.

In this work, the Hoechst fluorochrome assay was utilized to study the effects of several damaging agents on the survival of RIF-1 and RIF-1 derived variant cells. The results obtained at high doses of treatment were questionable due to high background fluorescence in each experiment. In some cases, DNA fluorescence counts at the highest treatment doses to cells were less than double the background fluorescence. This problem could not be overcome without exceeding the cell seeding capacity per well. Also the Hoechst fluorochrome assay does not discriminate between cells that have maintained their full reproductive capacity (which has been defined as cell survival (Hall 1994)) and those cells which may be physically intact, able to make proteins and synthesize DNA, but have lost the ability to divide indefinitely and produce a large number of progeny. In the context of future clinical applicability, an assay that does not distinguish between non dividing and dividing cancer cells may not be ideal. Results in the present study have been presented as preliminary work. Further research examining survival of RIF-1 and RIF-1 derived variant cells following treatment with damaging agents such as JM2929-PDT should utilize the clonogenic survival assay, where background levels are not a factor, and cell survival is based on the replicative capabilities of tumor cells.

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CHAPTER 3

SENSITIVITY OF MURINE FIBROSARCOMA CELLS TO PHOTODYNAMIC THERAPY MEDIATED BY THE RUTHENIUM PHTHALOCYANINE JM2929, UV LIGHT, GAMMA RADIATION, AND PHOTOFRIN-MEDIATED SENSITIZATION TO GAMMA-RAYS

ABSTRACT

We have previously reported an increased resistance of a murine fibrosarcoma cell variant, RIF-8A, to Photofrin-mediated photodynamic therapy (Ph-PDT) compared to parental RIF-1 cells. In the current work we have examined the clonogenic survival of the RIF-1 and RIF-8A cells following UV, gamma-rays and PDT mediated by the Ruthenium (II) phthalocyanine-bis-(triphenylphosphine-*m*-monosulfonate) potassium salt (JM2929). We show a cross resistance of RIF-8A cells to UV, but not to JM2929-mediated PDT (JM2929-PDT) or gamma rays. RIF-8A and RIF-1 cells showed a similar sensitivity to JM2929-PDT, whereas RIF-8A cells showed an increased sensitivity to gamma-rays compared to RIF-1 cells. We also examined the sensitivity to gamma rays for two other murine fibrosarcoma cell lines, RIF-P16CL8 and CPR-C1, previously reported to have increased resistance to Ph-PDT. Results show cross resistance to gamma rays for CPR-C1 cells but not for RIF-P16CL8 cells. This result suggests that the mechanism of Ph-PDT resistance developed in the RIF-8A cells differs from that in the CPR-C1 and RIF-P16CL8 cells. We also examined the clonogenic survival of RIF-8A and RIF-1 cells following gamma rays in the presence of either Photofrin or JM2929. RIF-1 cells, but not RIF-8A cells, showed reduced survival when gamma-irradiated in the presence of Photofrin, whereas gamma-irradiation in the presence of JM2929 had no effect on the survival of RIF-1 and RIF-8A cells. These results are thought to reflect a difference in the spectrum of cellular damage induced by Ph-PDT compared to JM2929-PDT. It is possible that Ph-PDT, but not JM2929-PDT, induces a "UV-

like" component of damage and/or there is some overlap in the pathways for the repair of UV and Ph-PDT damage, but not JM2929-PDT and ionizing radiation damage in RIF-1 and RIF-8A cells.

INTRODUCTION

Photodynamic Therapy (PDT) is an actively developing field in cancer treatment, currently in Phase III clinical trials for a number of human cancers (Dougherty and Marcus 1992, Larsen 1993, Lowdell 1994). PDT utilizes the localized delivery of light to activate a photosensitizing drug, which is selectively retained by tumour tissue (Manyak et al. 1988, Dougherty and Marcus 1992, Lowdell 1994, Henderson and Dougherty 1992). Although this treatment modality is undergoing clinical trials, the mechanism(s) of PDT cytotoxicity is not yet fully understood.

The photodynamic process requires three simultaneously present components for cytotoxicity namely a sensitizer, light, and oxygen. PDT involves the excitation of a photosensitizing drug by light energy to create singlet oxygen. Singlet oxygen molecules cause cytotoxic damage within tumour cells in vitro, and also in the tumour vasculature, in the case of in vivo PDT treatment (Moan et al. 1979, Pass 1993, Gomer et al. 1989, Henderson and Dougherty 1992). In vitro effects following PDT include membrane damage (Kessel 1977, Moan et al. 1989), mitochondrial damage (Gomer et al. 1988, Berns et al. 1982, Hilf et al. 1987), and DNA damage (Kvam and Stokke 1994, Gomer 1980, Fiel et al. 1981).

In vitro PDT research has taken advantage of a number of cell lines that exhibit varying PDT responses. These cell lines, along with new photosensitizers, have been used by several investigators in order to examine the mechanism of PDT cytotoxicity. Photofrin is the most common porphyrin photosensitizer to date which is already in the process of Phase

III clinical trials (Dougherty 1993). Several Ph-PDT resistant cell lines have been developed (Luna and Gomer 1991, Singh et al. 1991) including those derived from the radiation induced fibrosarcoma cell line (RIF-1) developed by Twentyman et al. (Twentyman et al. 1980). The RIF-1 tumor is a suitable tumor model system for studies in which clonogenic survival is compared to growth delay and tumor control following various forms of treatment including PDT. RIF-1 tumor cells have a satisfactory plating efficiency of 25% from in vivo to in vitro. The RIF-1 tumor is minimally immunogenic and does not metastisize from the intradermal injection site. Chromosome analysis has shown that both diploid and tetraploid tumor cells exist within the RIF-1 tumor (Twentyman et al. 1980). New photosensitizers under examination for their use in PDT include nile blue derivatives (Lin et al. 1993); metallophthalocyanines such as aluminum phthalocyanines with varying degress of sulfonation (Agarwal et al. 1992, Ben Hur et al. 1991, Ben-Hur et al. 1991, Bown et al. 1986, Moan et al. 1992), zinc-phthalocyanines (Daraio et al. 1991, Fingar et al. 1993, Obochi et al. 1993) JM2929, the ruthenium monosulphonate examined in the current work; and most recently carbodiimide analogs of bacteriochlorins (Pandey et al. 1989, Pandey et al. 1996).

Luna and Gomer (1991) reported the isolation of Ph-PDT resistant RIF-1 variants which exhibited a stable phenotpe and could be used in studies designed to define PDT mechanisms of action (Luna and Gomer 1991). Ph-PDT resistant clones from two different porphyrin incubation periods, namely RIFP16Cl4, RIFP16CL8, RIFP1CL1 and RIFP1CL5, had increased protein content, larger cellular size compared to RIF-1G (parental RIF-1 cells used by Luna and Gomer) parent cells, similar in vitro growth rates to the RIF-1G parent, modest

resistance to ionizing radiation, and in the case of one variant, RIF-P16CL4, increased sensitivity to hyperthermia compared to RIF-1G parent cells (Luna and Gomer 1991). None of the RIF-1 variants exhibited a multidrug resistant phenotype, nor did they have altered porphyrin uptake levels. The RIF-1G parental cells and Ph-PDT resistant variants showed comparable basal levels of antioxidant enzymes, reduced glutathione and stress proteins. However there was an increase in the number of cells required to form tumor growth in 50% of the inoculated animals for the Ph-PDT resistant cells. The CPR-C1 cell line was developed by Luna and Gomer by further repeated exposure of RIF-P16CL8 cells to Ph-PDT. This cell line has been shown to have higher levels of Ph-PDT resistance than the RIF-P16CL8 cells (Luna personal communication).

Another such Ph-PDT resistant cell line is the RIF-8A variant. RIF-8A cells were derived from RIF-1 parent cells by repeated Ph-PDT treatment followed by regrowth from single colonies (Singh et al. 1991). The RIF-8A cells have been previously reported to have significantly increased survival to Ph-PDT compared to RIF-1 cells. The average ratio, D_{10}(RIF-8A)/D_{10}(RIF-1) for 2.7 x 10^3 J/m^2 light dose in six independent experiments was found to be 1.8 ± 0.4 (Singh et al. 1991). No adriamycin cross resistance was seen in the Ph-PDT resistant cells (Singh et al. 1991). This result was interpreted to imply a different mechanism of Ph-PDT resistance compared to the classical multidrug resistance or pleiotropic resistance mechanism. Fluorescence flow cytometry and spectrofluorometry measurements showed no difference in Photofrin uptake between RIF-1 and RIF-8A cells (Singh et al. 1991). RIF-8A cells contain higher ATP pool levels and have higher levels of succinate dehydrogenase activity compared to RIF-1 cells. The RIF-8A cells have also been

shown to have smaller mitochondria which stain more densely and display higher cristae density compared to RIF-1 cells (Sharkey et al. 1992). Although the majority of RIF-8A cells contained polyploides of 120 chromosomes, there was an inconsistency in karyotype. It is thought that these characteristics may be related to the Ph-PDT resistance seen in RIF-8A cells (Sharkey et al. 1993). In 1994, Moorehead et al. reported that the RIF-8A cells showed a cross resistance to cisplatin (Moorehead et al. 1994) and a significant decrease in cisplatin-DNA adduct levels compared to the RIF-1 parent cells. Rhodamine 123 accumulation was shown to be 3 and 3.6 fold less in the plasma and mitochondrial membranes respectively in RIF-8A cells compared to parental RIF-1 cells. Moorehead et al. (1994) concluded that alterations in the plasma and/or mitochondrial membrane potentials may provide the RIF-8A cells with a survival advantage when challenged with PDT or cisplatin. Di Prospero et al. (1996) have reported that the capacity of UV-irradiated cells for viral DNA synthesis was greater for RIF-8A cells compared to RIF-1 cells suggesting a cross-resistance of RIF-8A cells to UV.

In the current work, the survival of RIF-1 and RIF-8A cells were examined following exposure to UV light, gamma rays, or PDT mediated by the novel Ruthenium phthalocyanine, JM2929. Results indicate a cross resistance of RIF-8A cells to UV light, but not to gamma-rays or JM2929-PDT. We also show a cross resistance of CPR-C1 but not RIF-P16CL8 cells to gamma rays. We also examined the potential of Photofrin and JM2929 to act as sensitizers to gamma-ray exposure. Results indicate that Photofrin can act as a sensitizer to gamma-ray exposure for RIF-1, but not RIF-8A cells, whereas JM2929 had no

effect on gamma ray survival of RIF-1 or RIF-8A cells over the range of photosensitizer and gamma-ray doses employed.

MATERIALS AND METHODS

Cells

RIF-1 cells were provided by Dr. B. Henderson, Roswell Park Memorial Institute, Buffalo, New York. RIF-8A cells were obtained from Dr. Gurmit Singh, Hamilton Regional Cancer Clinic and McMaster University, Dept. of Pathology, Hamilton, ON. RIF-1G, RIF-P16 and CPR-C1 were obtained from Dr. C. Gomer at the Clayton Ocular Oncology Centre, Children's Hospital of Los Angeles, Los Angeles, CA. All cells were grown in monolayer and maintained in Eagles α -minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (100 μg/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B; Gibco BRL), (growth media) and incubated at 37°C (5% CO_2/95% air in a humid environment).

Photosensitizers and Light Source

Photofrin was obtained from Quadralogic Technologies Inc., Vancouver, B.C. Canada. JM2929 was obtained from Johnson Matthey, Inc., PA, USA. Both sensitizers were diluted to concentrations between 5-20 mg/mL in growth media. Cells were pretreated with photosensitizers 18 hours prior to red light exposure on a 100cm X 50cm light diffusing surface illuminated by a bank of fluorescent tubes (Philips type TL/83) filtered with red acetate filters (Roscolux, No.19, Rosco, CA) to give wide band illumination above 585 nm. The energy fluence rate was approximately 9.2 J/m^2/sec. Exposure for 5 minutes resulted in incident energy fluence of approximately 2 760 J/m^2.

Colony Forming Assay

All colony forming assays were performed in 6 well plastic petri dishes. Appropriate numbers of cells were seeded in order to count between 100-200 colonies per well post treament. Plates were incubated at 37°C (5% CO_2/95% air in a humid environment) for 4 days prior to staining with methylene blue (5% methylene w:v in 70% methanol and 30% dH_20) and only colonies greater than 20 cells were counted. In each experiment, colony counts for each cell treatment were the average of triplicate determinations.

Photofrin or JM2929 mediated PDT

Appropriate number of cells were seeded in 6 well plastic petri dishes and allowed to adhere for 6 hours. Both sensitizers were diluted to concentrations between 5-20 mg/mL in growth media. Cells were treated with photosensitizer and incubated for 18 hours. Growth medium containing the photosensitizer was then aspirated from the wells and replaced with prewarmed growth media immediately prior to illumination with red light. Cells were then incubated at 37°C (5% CO_2/95% air in a humid environment) for four days prior to staining for colony counting. All procedures involving photosensitizer were carried out under minimal ambient lighting conditions.

UV-Irradiation of Cells

All cells were seeded in 6 well plastic petri dishes for colony forming assays. Following a six hour attachment period, growth media was aspirated and replaced with 1mL of pre-warmed phosphate buffer saline (PBS) (140 mM NaCl, 2.5 mM Kcl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4) prior to UV irradiation. UV light emitting primarily at 254 nm was delivered using a General Electric germicidal lamp (model G8T5). All cells were exposed at a fluence rate of 1 $J/m^2/sec$ (J-255 Shortwave UV meter, Ultraviolet Products, San Gabriel, California). Cells were incubated for four days prior to staining. UV exposure to cells varied from 5-40 J/m^2.

Gamma-radiation of Cells

All cells were seeded in 6 well plastic culture dishes for colony forming assays. Following a six hour attachment period, plates were placed into sterile tupperware containers to avoid contamination. ^{60}Co irradiation was delivered by a Theratron 80 Cobalt-D machine (supplied by Atomic Energy of Canada) at dose rates ranging from 65.03 cGy/min to 61.63 cGy/min. Dose rates were calculated for each experiment using initial ionizing chamber measurements made according to AAPM TG21 standardized protocol (McGregor 1996, private communication). Shutter correction time was 0.01 minutes. Source to surface distance was 80 cm for each experiment with an irradiation field of 30cm X 30 cm. Doses given to cells varied from 50 cGy to 1000 cGy.

Gamma-radiation with Photosensitizer

In the case of pretreatment with a sensitizer, cells were incubated with 20 μg/mL photosensitizing drug for 18 hours. Growth media from the wells was then aspirated and replaced with prewarmed fresh growth media prior to gamma radiation. Gamma radiation pararmeters and doses were as described above. Experiments were conducted under minimal ambient light conditions.

RESULTS

Sensitivity of RIF-1 and RIF-8A cells to Photofrin-PDT and JM2929-PDT

RIF-1 and RIF-8A cells were treated with varying doses of Ph-PDT. No dark toxicity was observed in RIF-1 or RIF-8A cells following a sensitizer dose of 20 μg/mL. No effect on survival in RIF-1 and RIF-8A cells was observed following a light dose of 2.76 x 10^3 J/m^2 light alone. Each individual experiment showed significantly increased resistance to Ph-PDT in RIF-8A cells compared to RIF-1 cells. Pooled results for the clonogenic survival of RIF-1 and RIF-8A cells following exposure to Photofrin mediated PDT are shown in Figure 1A. An increased resistance was observed in RIF-8A cells compared to RIF-1 cells as reported previously (Singh et al. 1991). RIF-1 and RIF-8A cells were also treated with JM2929-PDT. Each individual experiment showed no significant difference in sensitivity to JM2929-PDT between RIF-1 and RIF-8A cells. Pooled results for clonogenic survival of RIF-1 and RIF-8A cells following exposure to JM2929-PDT are shown in Figure 1B. Results for each drug dose were plotted using the linear quadratic equation ($SF=e^{-(\alpha x+\beta x^2)}$) (where SF is the surviving fraction of cells). D_{10} (dose required to reduce colony survival to 10%) values were extrapolated for a number of independent experiments. The average ratio of D_{10}(RIF-8A cells)/D_{10}(RIF-1 cells) for a light dose of approximately 2.76 x 10^3 J/m^2 in three independent experiments was 0.90 ± 0.13.

Sensitivity of RIF-1 and RIF-8A cells to UV

Recent studies have shown a cross resistance of the RIF-8A cell line to Ph-PDT and other cytotoxic agents including cisplatin (Moorehead et al. 1994) and UV (Di Prospero 1996). UV cross resistance of RIF-8A cells was examined using the viral capacity assay. The viral capacity assay measures the ability of cells, which have been treated with a chemical or physical agent, to support viral growth. It was therefore of interest to examine the sensitivity of RIF-1 and RIF-8A cells following UV exposure by the colony forming assay. Pooled results for the survival of RIF-1 and RIF-8A cells following UV exposure are shown in Figure 2. It can be seen that the Ph-PDT resistant RIF-8A cells show a cross-resistance to UV exposure. D_{10} values were obtained from the best linear quadaratic fit for each cell line in three independent experiments. The average ratio, D_{10}(RIF-8A cells)/D_{10} (RIF-1 cells) of three independent experiments was 1.25 ± 0.14.

Sensitivity of RIF cells to Gamma-radiation

RIF-P16 and CPR-C1 cells developed by Luna and Gomer (1991) have previously been described to have varying resistance to Ph-PDT (Luna and Gomer 1991, Singh unpublished observation). Two RIF-P16 clones, namely RIFP16CL4 and RIFP16CL8, exhibited a modest resistance to ionizing radiation (Luna and Gomer 1991). It was therefore considered of interest to examine the gamma-ray sensitivity of RIF-1 and RIF-8A cells in comparison to that observed for RIF-P16CL8 and CPR-C1 cells derived by Luna and Gomer (1991). RIF-1G, RIF-P16CL8 and CPR-C1 cells as well as RIF-1 and RIF-8A cells were examined for survival following gamma-rays. Results of Figure 3 show that over a

dose range of 0-10 Gy, a significant increase in resistance to radiation was seen in the CPR-C1 cell line compared to the parental RIF-1G cell line, but not by RIF-P16CL8 cells compared to the parental RIF-1G cell line. Survival curves from individual experiments were fitted to a linear quadratic equation and D_{10} values extrapolated. The average ratio of D_{10}(CPR-C1)/D_{10}(RIF-1G) and D_{10}(RIF-P16CL8)/D_{10}(RIF-1G) from three independent experiments was 1.52 ± 0.15 and 0.88 ± 0.07 respectively.

The RIF-1 tumor cells originated from gamma radiation induced fibrosarcoma cells. The results shown in Figure 4 indicate that the Ph-PDT resistant RIF-8A cells do not have a cross resistance to gamma radiation. In fact RIF-8A cells showed significantly increased sensitivity to gamma-rays compared to RIF-1 parent cells. Survival curves from independent experiments were fitted to a linear quadratic equation and D_{10} values extrapolated. The average ratio of D_{10}(RIF-8A)/D_{10}(RIF-1) for six independent experiments was 0.65 ± 0.04.

Examination of Gamma-ray Sensitization with Photofrin and JM2929

There are some recent reports suggesting that photosensitizers in combination with ionizing radiation treatment can act as radiosensitizers under aerobic conditions (Luksiene et al. 1994, Berg et al. 1995). Figures 5 and 6 show that a drug concentration of 20 mg/mL of Photofrin results in a significant increase in sensitivity to gamma-rays for RIF-1 cells but not RIF-8A cells. Survival curve from individual experiments were fitted to a linear quadratic equation and D_{10} values extrapolated. The average ratio of D_{10} (RIF cells + Photofrin)/D_{10}(RIF cells + no drug) of three independent experiments was 0.88 ± 0.03 for

RIF-1 cells and 1.03 ± 0.12 for RIF-8A cells. This result indicates that Photofrin can act as a radiosensitizer in RIF-1 cells.

Figures 5 and 6 also show the effects of JM2929 on the gamma ray survival of RIF-1 and RIF-8A cells. It can be seen that JM2929 had no influence on gamma-ray sensitivity for either RIF-1 or RIF-8A cells. Survival curves from individual experiments were fitted to a linear quadratic equation and D_{10} values extrapolated. The average ratio of D_{10}(RIF cells + JM2929)/D_{10}(RIF cells + no drug) of three independent experiments was 1.01 ± 0.02 for RIF-1 cells, and 1.0 ± 0.06 for RIF-8A cells.

Figure 1: Colony Forming Ability of Ph-PDT or JM2929-PDT treated RIF-1 and RIF-8A cells.

Shows pooled results from three independent experiments for survival of RIF-1 (-■-) and RIF-8A (-●-) cells following Ph-PDT (A) and JM2929-PDT (B). Each survival point is the arithmetic mean ± standard error.

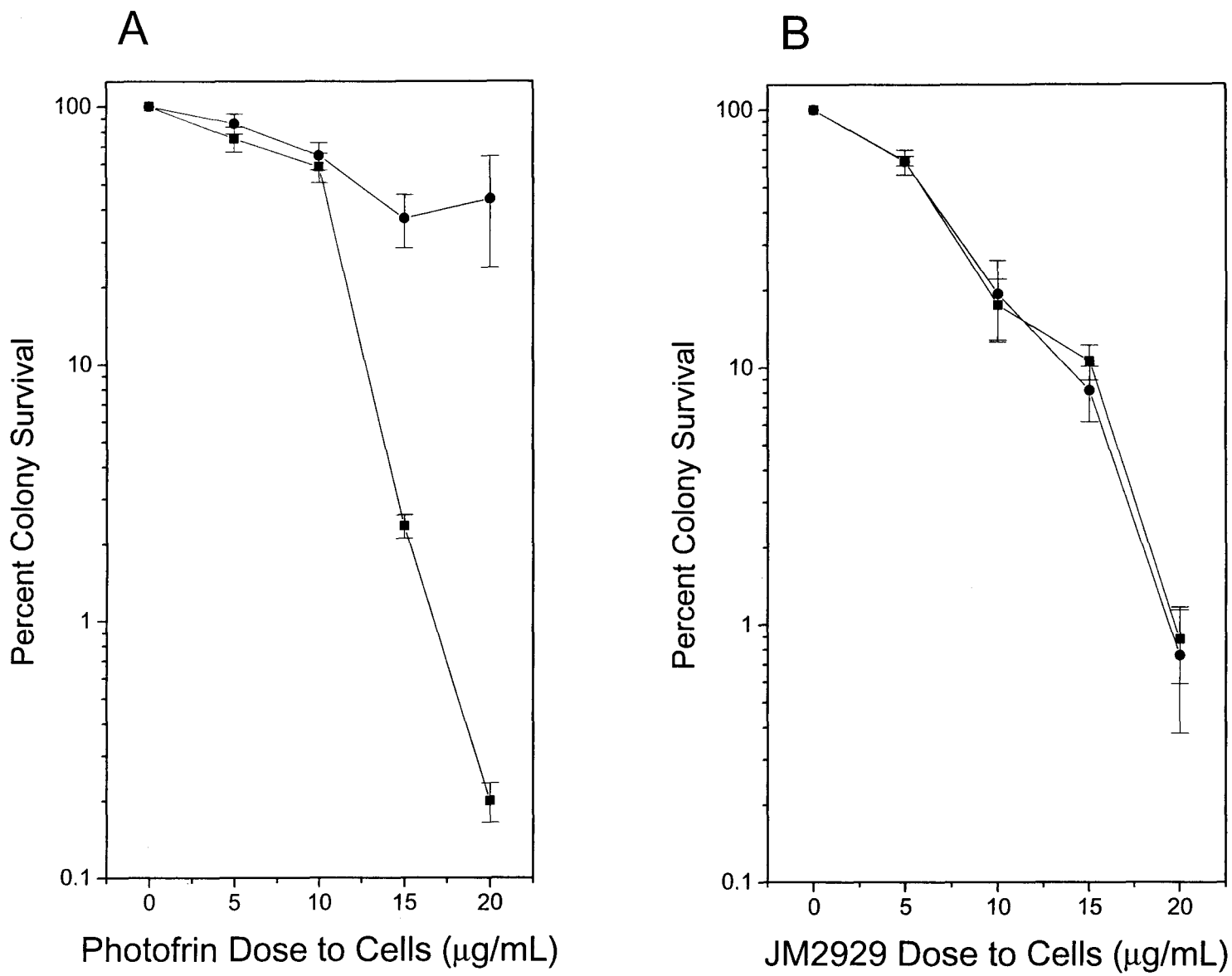

A
100
10
1
0.1
Percent Colony Survival
0
5
10
15
20
Photofrin Dose to Cells (μg/mL)
B
100
10
1
0.1
Percent Colony Survival
0
5
10
15
20
JM2929 Dose to Cells (μg/mL)

Figure 2: Colony Forming Ability of UV treated RIF-1 and RIF-8A cells.

RIF-1 (-■-) and RIF-8A (-●-) cells were treated with UV irradiation. Shows pooled results from three independent experiments. Each survival point is the arithmetic mean ± standard error.

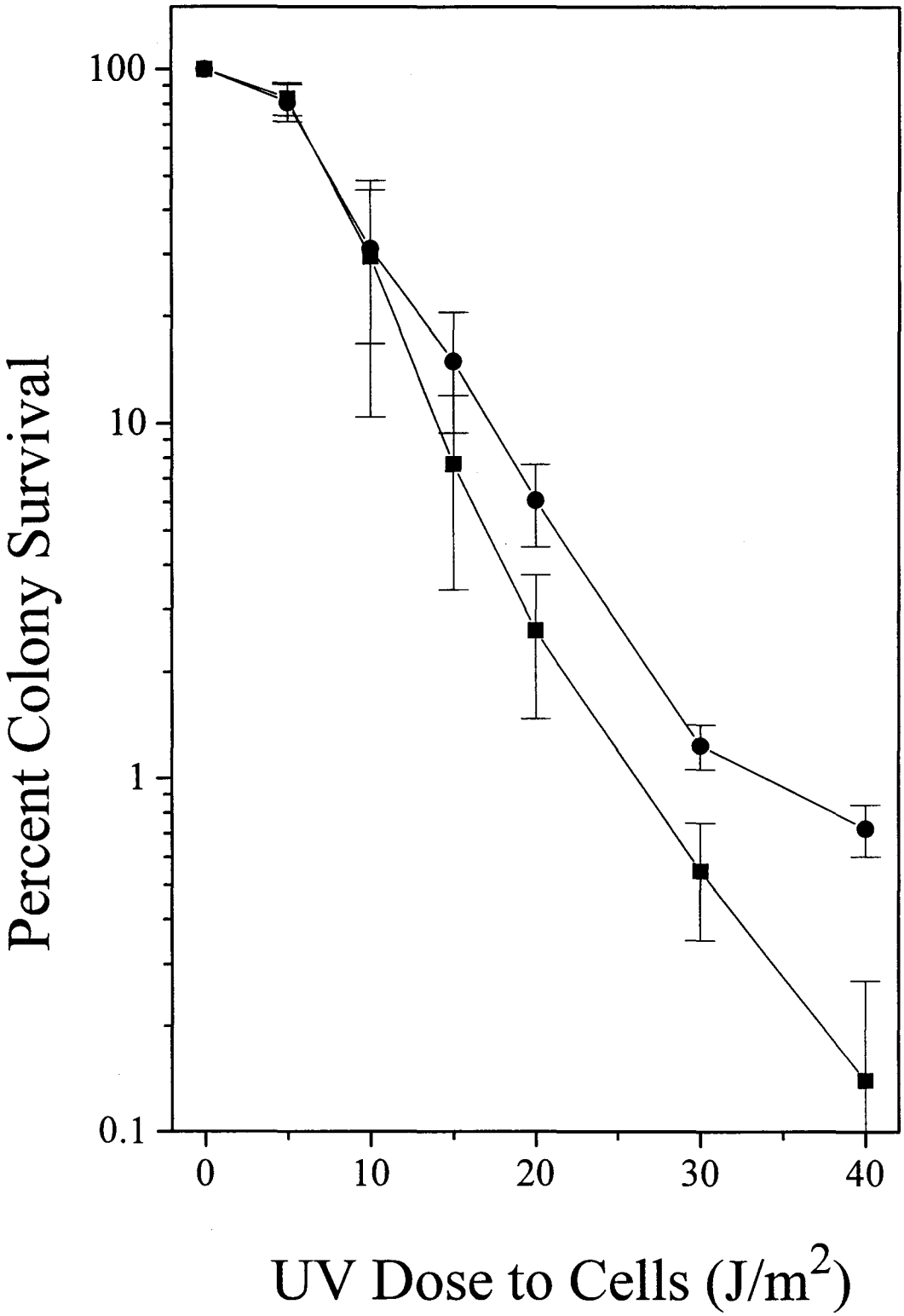

100
10
1
0.1
Percent Colony Survival
0
10
20
30
40
UV Dose to Cells (J/m2)

Figure 3: Colony Forming Ability of RIF-1G, RIF-P16, and CPR-C1 cells following exposure to gamma- rays.

Shows pooled results of RIF-1G (-■-), RIF-P16CL8 (-●-), and CPR-C1 (-▲-) cells treated with gamma-rays from three independent experiments. Each survival point is the arithmetic mean ± standard error.

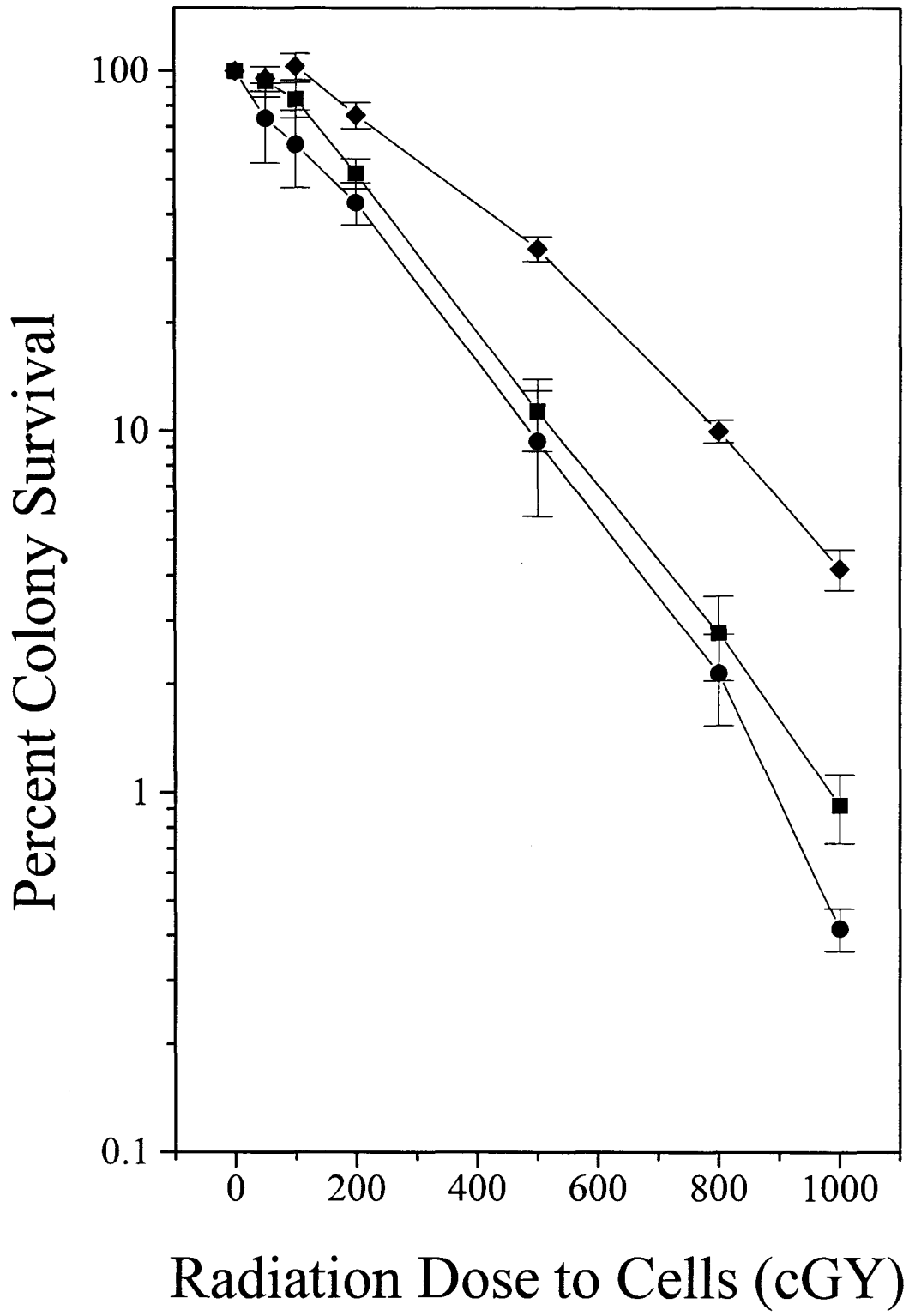

Percent Colony Survival
100
10
1
0.1
0
200
400
600
800
1000
Radiation Dose to Cells (cGY)

Figure 4: Colony Forming Ability of RIF-1 and RIF-8A cells following treatment with gamma-rays.

RIF-1 (-■-) and RIF-8A (-●-) cells were treated with gamma-rays. Shows pooled results from three independent experiments. Each survival point is the arithmetic mean ± standard error.

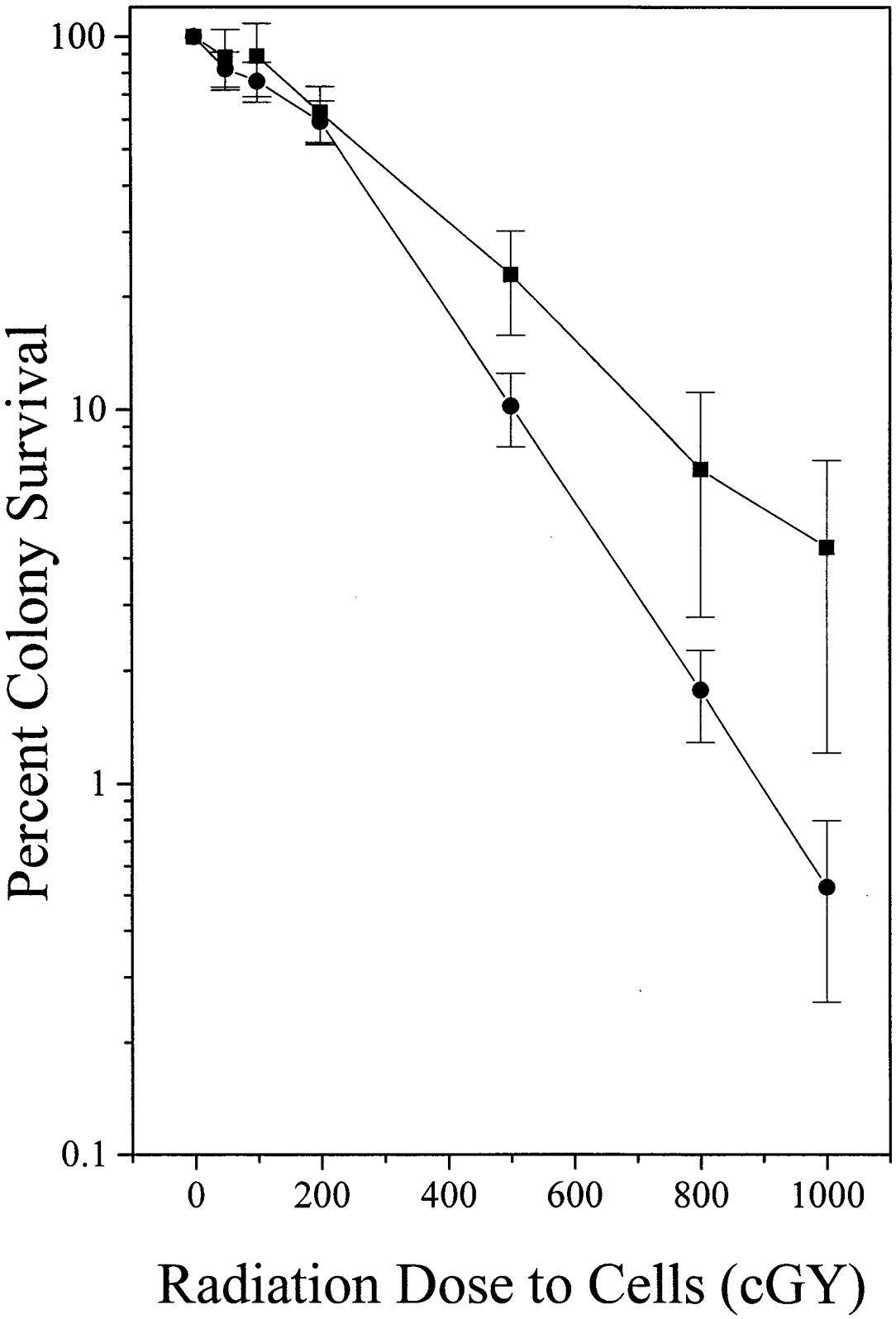

100
10
1
0.1
Percent Colony Survival
0
200
400
600
800
1000
Radiation Dose to Cells (cGY)

Figure 5: Colony Forming Ability of RIF-1 cells following gamma-ray sensitization with Photofrin or JM2929.

RIF-1 cells were pretreated with no drug (-■-), 20μg/mL Photofrin (-◆-), or 20μg/mL JM2929 (-●-) for 18 hours prior to gamma radiation. Shows pooled results from three independent experiments. Each survival point is the arithmetic mean ± standard error of triplicate determinations.

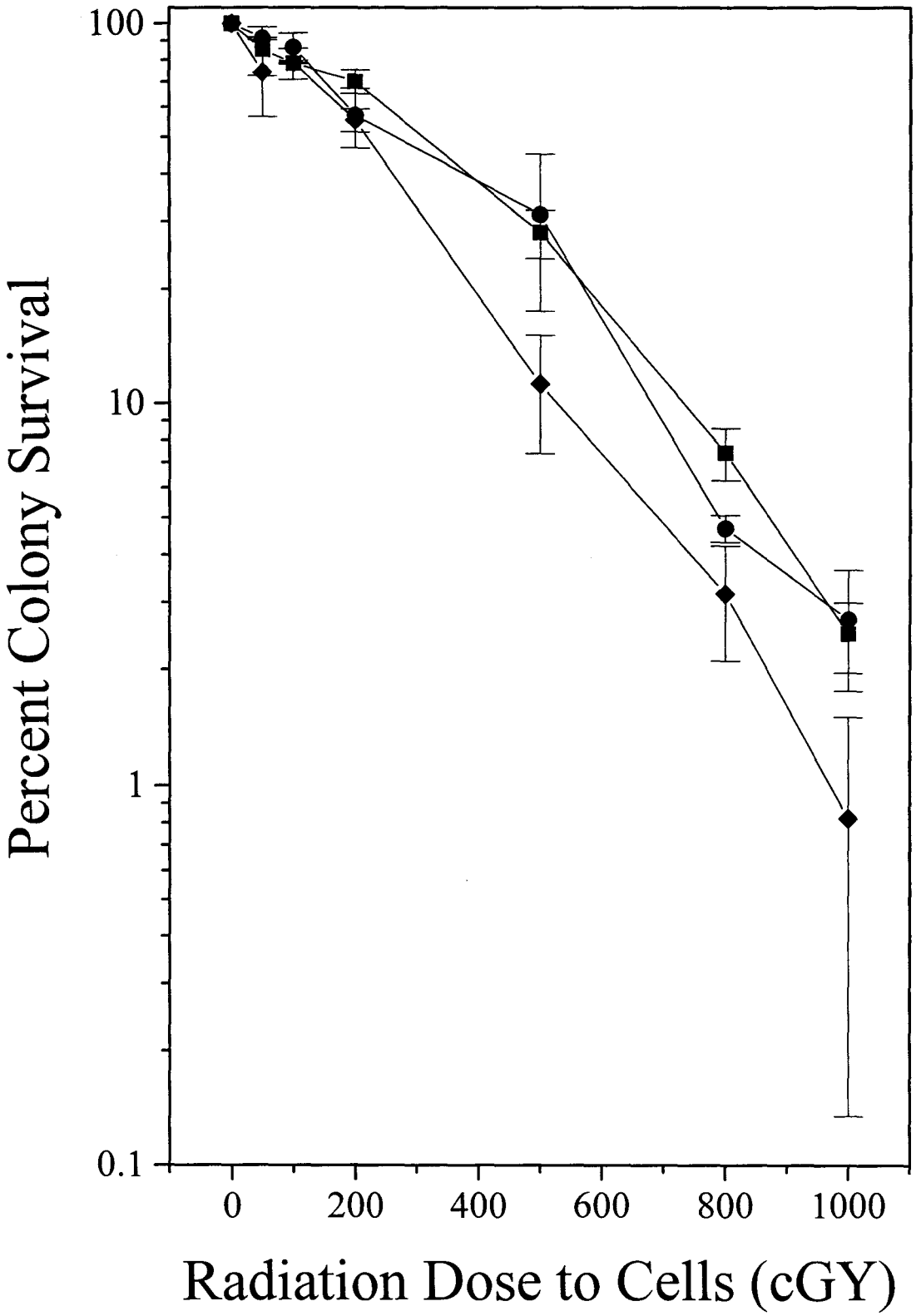
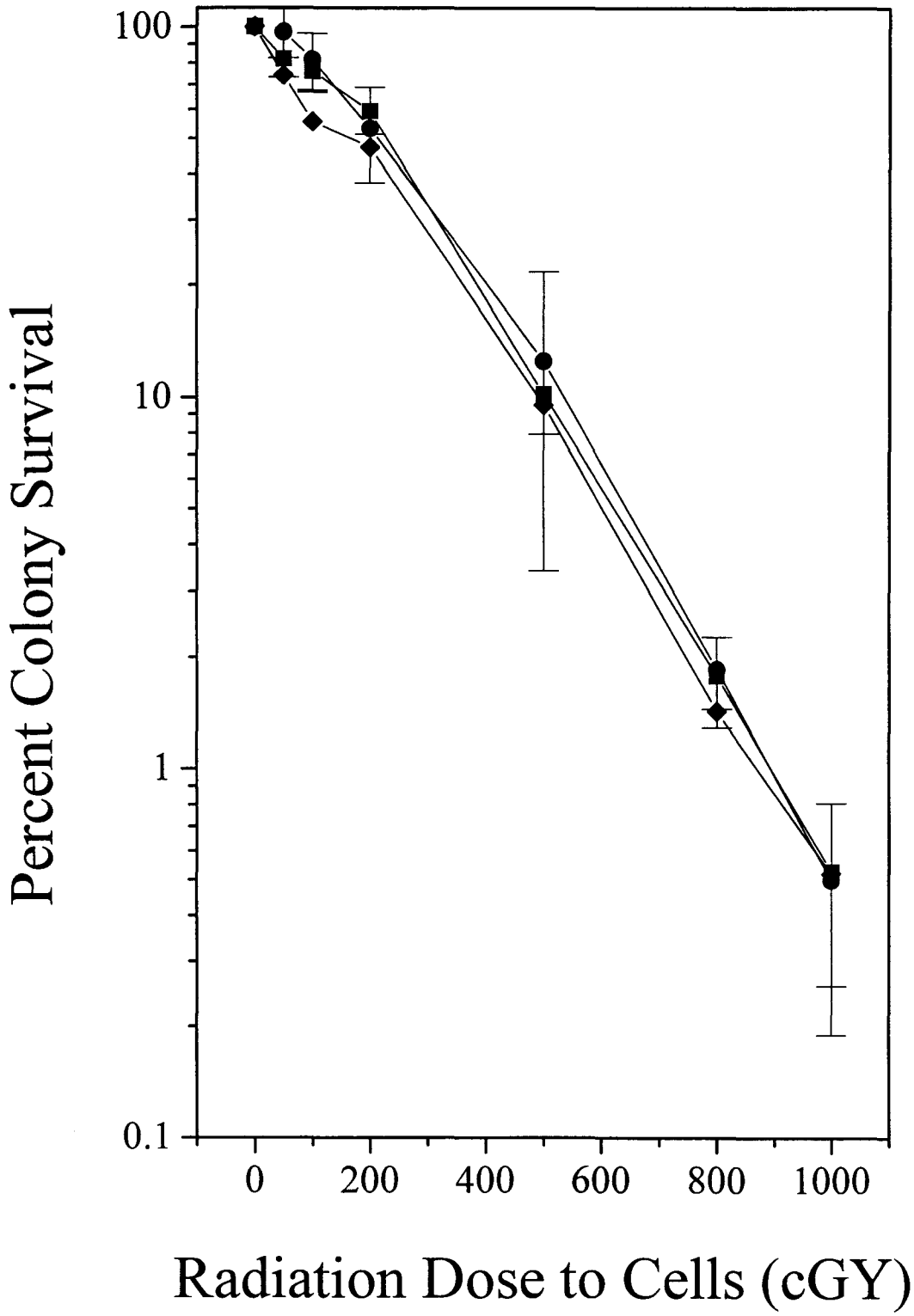

100
10
1
0.1
Percent Colony Survival
0
200
400
600
800
1000
Radiation Dose to Cells (cGY)

Figure 6: Colony Forming Ability of RIF-8A cells following gamma-ray sensitization with Photofrin or JM2929.

RIF-8A cells were pretreated with no drug (-■-), 20μg/mL Photofrin (-◆-), or 20μg/mL JM2929 (-●-) for 18 hours prior to gamma radiation. Shows pooled results from three independent experiments. Each survival point is the arithmetic mean ± standard error.


Percent Colony Survival
100
10
1
0.1
0
200
400
600
800
1000
Radiation Dose to Cells (cGY)

DISCUSSION

RIF-8A cells have been previously characterized to have increased resistance to Ph-PDT compared to RIF-1 cells (Singh et al. 1991). Electron micrographs showed smaller mitochondria with higher cristae density in RIF-8A cells compared to RIF-1 cells (Sharkey et al. 1993). As well, RIF-8A cells showed increased levels of ATP production, increased succinate dehydrogenase activity, and higher chromosomal counts compared to RIF-1 cells. (Sharkey et al. 1992, Sharkey et al. 1993, Twentyman et al. 1980). Cross resistance to cisplatin and UV has also been observed in Ph-PDT resistant RIF-8A cells (Moorehead et al. 1994, Di Prospero et al. 1996)

JM2929 is a novel photosensitizer which is composed of a monosulphonated phthalocyanine ring chelated with a Ruthenium ion. The wavelength of excitation for JM2929 is 650nm. RIF-1 and RIF-8A cells were treated with JM2929-PDT to compare survival responses of these cells to those observed following Ph-PDT. No significant difference in colony formation was found between RIF-1 and RIF-8A cells treated with JM2929-PDT. Ratio of D_{10} values (D_{10} RIF-8A cells /D_{10} RIF-1 cells) obtained from the linear quadratic fit for each cell line was 0.90 ± 0.13. It has been suggested that one of the mechanisms for Ph-PDT resistance seen in the RIF-8A cells may the mitochondrial morphological and functional differences observed in RIF-8A compared to RIF-1 cells (Sharkey et al. 1993). However, these differences do not appear to result in enhanced survival of RIF-8A cells compared to the RIF-1 parent cells following JM2929-PDT. Phthalocyanines and porphyrins are both thought to elicit cellular damage primarily by

producing singlet oxygen (Daraio et al. 1991, Biade et al. 1992). The subcellular targets for JM2929-PDT have not yet been defined but like porphyrins, monosulfonated phthalocyanines can cause damage to plasma membranes, subcellular organelle membranes such as mitochondria, lysosomes, nuclei, and nuclear DNA (Evans et al. 1989, Zaidi et al. 1993, Ben-Hur et al. 1991, Ben-Hur et al. 1987, Gomer et al. 1988 Moan et al. 1992). However, the target within the cell which is critially damaged by the photodynamic action of phthalocyanines may differ from that seen by porphyrin mediated PDT damage. Absence of cross resistance of Ph-PDT resistant RIF-8A cells to JM2929-PDT suggests that the cytotoxic damage induced by Ph-PDT and JM2929-PDT has different subcellular targets.

The DNA damaging effects of UV irradiation are known to include the formation of pyrimidine dimers and the (6-4) photoproduct (Friedberg et al. 1995). Di Prospero et al. (1996) reported an increased capacity for adenovirus DNA synthesis in UV treated Ph-PDT resistant RIF-8A cells compared to UV treated RIF-1 cells (Di Prospero et al. 1996). The viral capacity assay uses viral DNA synthesis as an indicator of cellular sensitivity to and recovery from cellualr damaging agents. Results of the present work also indicate a cross resistance of Ph-PDT resistant RIF-8A cells to UV light over the exposures employed. D_{10} values for colony survival following UV exposure were significantly different between the two cells lines (D_{10} ratio (RIF-8A cells/RIF-1 cells) of 1.25 ± 0.14). Cross resistance seen between Ph-PDT and UV in the RIF-8A cell line suggests that there may be similarities in the components of damage and/or similarities in the repair pathways of the two treatment modalities.

Ionizing radiation is known to cause DNA strand breaks (Hall 1994). The RIF tumor model system was derived from a radiation induced fibrosarcoma after repeated exposure to gamma radiation of the hindlimb of a C3H/Km inbred mouse (Twentyman et al. 1980). As a result, RIF-1 cells are highly resistant to radiation (Twentyman et al. 1980, Waldow et al. 1987). In this study, RIF-1 and RIF-8A cells were exposed to gamma radiation for the purpose of comparing their clonogenic survival. Ph-PDT resistant RIF-8A cells were shown to have increased sensitivity to gamma radiation compared to their RIF-1 parent cells. D_{10} values obtained from each survival curve indicate a significant difference in colony forming ability between gamma-ray treated RIF-1 and RIF-8A cells with a D_{10} ratio (RIF-8A cells/RIF-1 cells) of 0.65 ± 0.04. This suggests that the cellular changes required for the RIF-8A cells to develop resistance to Ph-PDT interferes with the ability of the cells to recover from the cellular damage caused by gamma radiation. This result may be of added interest in the clinical setting. It suggests that Ph-PDT resistant tumors may have increased sensitivity to radiation in which case patient therapy can be altered.

The increase in sensitivity seen in the RIF-8A cells upon ionizing radiation treatment is interesting in contrast to results obtained for Ph-PDT resistant RIF-P16CL8 and CPR-C1 cells developed by Luna and Gomer (1991). However D_{10} values for gamma irradiated RIF-1 and RIF-1G cells were 773.33 Gy $\pm$ 66.48 and 535 Gy $\pm$ 55.08 respectively, suggesting a significant difference in clonogenic survival following exposure to ionizing radiation between the two RIF-1 parent cell lines. Different in vitro maintenance and frequency of in vivo repassage through mice may be related to the differences in gamma-ray sensitivities seen between the RIF-1 and RIF-1G cells reported here. The RIF-P16CL8 cell

line has previously been reported to show a modest resistance to ionizing radiation (Luna et al. 1991). However for up to 10 Gy, which was the maximum irradiation dose used in the present study, Luna and Gomer (1991) did not see a significant increase in survival of the RIF-P16CL8 compared to parental RIF-1G cells (Luna and Gomer 1991). This is consistent with results presented in Figure 3. Figure 3 also shows a significantly increased level of resistance to gamma rays by CPR-C1 cells. (D_{10} ratio of CPR-C1 cells/RIF-1G cells was 1.52 $\pm$ 0.15). Preliminary results from our laboratory using Hoechst fluorochrome assay indicate that RIF-P16CL8 and CPR-C1 cells, unlike RIF-8A cells, are cross resistant to JM2929-PDT treatment. These results suggest that the cellular changes required for RIF-8A cells compared to RIF-P16CL8 and CPR-C1 cells to become resistant to Ph-PDT are different, or that additional changes have occurred in one or all cell lines that effect gamma-ray and JM2929-PDT sensitivity. This suggests more than one mechanism of induced resistance to Ph-PDT by RIF cells.

There are some recent reports suggesting that photosensitizers in combination with radiation treatment can act as radiosensitizers under aerobic conditions (Luksiene et al. 1994, Berg et al. 1995). In 1994, Luksiene et al. reported that concentrations more than 1mM of 5-Aminolevulinic acid, which induced endogenous protoporphyrin IX, had modifying effects on X-rays and slightly radiosensitized WiDr (human primary adenocarcinoma of rectosigmoidal colon) cells in culture. The ability of photosensitizers to serve as radiosensitizers may be dependent on the dose of ionizing radiation (O'Hara et al. 1989), the dose of photosensitizer (Luksienne et al. 1994), the type of sensitizer (Moan and Petterson 1981), and the dose rate (Berg et al. 1995). It was considered of importance to

examine the combination treatment of gamma-rays and photosensitizers simultaneously, using Photofrin and JM2929 in RIF-1 and RIF-8A cells. In the conventional sense, this was not combined PDT and radiation therapy. In this case, photosensitizer was not exposed to a light source of the appropriate wavelength at any time during gamma-ray treatment. A significant radiosensitizing effect was observed with Photofrin, but not JM2929, in gamma-ray treated RIF-1 cells. D_{10} ratios of Photofrin and JM2929 sensitized RIF-1 cells compared to RIF-1 cells radiated in the absence of photosensitizers was 0.88 ± 0.03 and 1.01 ± 0.02 respecitively. The Ph-PDT resistant RIF-8A cells were shown to have increased sensitivity to gamma radiation compared to RIF-1 cells, but interestingly were not further radiosensitized when irradiated in the presence of Photofrin (D_{10} ratio (Ph-treated cells/non-treated cells) of 1.03 ± 0.12). JM2929 also appeared to have no significant sensitization or protection effect on gamma irradiated RIF-8A cells (D_{10} ratio (JM2929-treated cells/non-treated cells) of 1.0 ± 0.06). The decreased survival seen in RIF-1 cells following gamma irradiation in the presence of Photofrin suggests that this photosensitizer may be of added benefit in the therapy of radioresistant tumors, or perhaps in radiation treatments of tumors unaccessible to PDT treatment.

Other investigators have also compared the effects of PDT, UV and ionizing radiation on mammalian tumor cells (Gomer et al. 1983, Evans et al. 1989). There is evidence to suggest a correlation between PDT, UV and radiation sensitivities in murine cell lines. There is a radiation resistant murine lymphoma cell line, L5178Y (LY-R) from which a radiation sensitive strain (LY-S) has been derived. The radiation sensitive LY-S strain, like RIF-8A cells, was also shown to be more resistant to UV irradiation than LY-R cells through

clonogenic studies (Beer et al. 1983). In 1989, Evans et al. reported on the increased cytotoxic sensitivity to chloroaluminum phthalocyanine and Photofrin mediated PDT in the radiation resistant L5178Y (LY-R) mouse lymphoma cells. Although there was no increased cytotoxic sensitivity of radiation resistant cells RIF-1 cells was seen for JM2929 phthalocyanine mediated PDT, this result correlates with the Ph-PDT clonogenic survival results observed for the RIF-1 and RIF-8A cells used in this work. The ionizing radiation sensitive LY-S cell strain, like RIF-8A cells showed cross resistance to UV and Ph-PDT, whereas radiation resistant LY-R cells, like RIF-1 cells, showed a cross-sensitivity to UV and Ph-PDT. The LY-R and LY-S cells differed in their DNA repair capabilities with the LY-R strain being deficient in the repair of UV induced pyrimidine dimers (Hagan et al. 1988) and the LY-S cells being deficient in the repair of DNA double stranded breaks (Evans et al. 1987). Evans et al. (1987) also reported a higher number of cytotoxic lesions in the LY-R strain, but a higher number of mutagenic lesions in the LY-S strain upon phthalocyanine mediated PDT treatment (Evans et al. 1987). They suggested that cytotoxic and mutagenic lesions differ and that their phthalocyanine mediated PDT sensitive strain was perhaps less efficient in the process involved in the repair of cytotoxic DNA damage. It may be suggested that RIF-1 and RIF-8A cells also differ in their DNA repair capabilities. RIF-1 cells, like LY-R cells, may be deficient in the repair of UV induced pyrimidine dimers and RIF-8A cells, like LY-S cells, may be deficient in the repair of DNA double stranded breaks. Further investigation is required to compare DNA repair differences between the RIF-1 and RIF-8A cells and to measure whether cytotoxic DNA lesion repair is less effecient in RIF-1 cells compared to RIF-8A cells following Ph-PDT.

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CHAPTER 4

ENHANCED REACTIVATION OF A UV-DAMAGED REPORTER GENE IN RADIATION INDUCED MURINE FIBROSARCOMA CELLS FOLLOWING PDT, UV, CISPLATIN AND HYPERTHERMIA

ABSTRACT

RIF-8A cells have been reported previously to show a cross-resistance to Photofrin mediated Photodynamic Therapy (Ph-PDT), cisplatin and UV light. However RIF-1 and RIF-8A cells show similar survival following JM2929 mediated PDT (JM2929-PDT) or hyperthermia treatment. In this work, host cell reactivation (HCR) and enhanced reactivation (ER) of a UV-damaged reporter gene were examined in the RIF-1 cell line and its Ph-PDT resistant variant RIF-8A. This was done using a UV damaged recombinant nonreplicating adenovirus based AdCA35*lacZ* virus expressing β-galactosidase (β-gal) from the *lacZ* reporter gene. HCR results indicate increased reactivation of a UV damaged reporter gene in untreated RIF-1 cells compared to untreated RIF-8A cells. ER was examined in RIF-1 and RIF-8A cells which had been treated with Ph-PDT, JM2929-PDT, UV, cisplatin or hyperthermia. Over the range of Ph-PDT and JM2929-PDT doses examined, and low doses of UV light (5 J/m^2), increased levels of reactivation of a UV damaged reporter gene were observed in RIF-1 cells compared to RIF-8A cells. Whereas, cisplatin pretreatment did not result in ER, and heat shock treatment (30 minutes heat shock at 43.5°C ± 0.25) resulted in decreased levels of reactivation of a UV-damaged reporter gene in both RIF-1 and RIF-8A cells. Enhanced expression of β-gal activity from a non-damaged reporter gene was also examined in RIF-1 and RIF-8A cells following pretreatment with Ph-PDT, JM2929-

PDT, UV, cisplatin and hyperthermia. Enhanced expression of β-gal activity from non-irradiated AdCA35*lacZ* was greater in RIF-8A cells following pretreatment with Ph-PDT. However results suggest no difference in enhanced expression of β-gal activity from non-irradiated AdCA35*lacZ* in RIF-8A cells compared to RIF-1 cells following JM2929-PDT, UV, cisplatin, or hyperthermia pretreatment, over the range of doses examined. These results suggest that the relation between survival, enhanced reactivation of a UV-damaged reporter gene, and enhanced expression of a non-damaged reporter gene varies in RIF-1 and RIF-8A cells depending on the cellular damaging agent employed. However increased expression of β-gal activity from a non-damaged reporter gene and decreased reactivation of a UV-damaged reporter gene in RIF-8A cells following Ph-PDT may be related to the Ph-PDT resistance observed in RIF-8A cells. Also, decreased levels of inducible repair of a UV damaged reporter gene in RIF-8A cells compared to RIF-1 cells following both Ph-PDT and UV pretreatments may suggest common mechanism(s) for Ph-PDT and UV resistance observed in RIF-8A cells.

INTRODUCTION

Ph-PDT resistant RIF-8A cells derived from radiation induced fibrosarcoma (RIF-1) cells have also shown a cross resistance to UV (Di Prospero et al. 1996, Roy et al. 1996) and cisplatin (Moorehead et al. 1994). Photofrin molecules accumulate in cellular membranes but have also been shown to cause DNA damage upon photoactivation (Gomer 1980, Gomer et al. 1988, Moan et al. 1989, Kvam and Stokke 1994, Fiel et al. 1981, Evensen et al. 1982, Dubbelman et al. 1982). UV and cisplatin also cause DNA damage (Freidberg et al. 1995). UV irradiation induces pyrimidine dimers and (6-4) photoproducts in DNA. Cisplatin forms adducts which cause the helical structure of DNA to bend (Friedberg et al. 1995). Altered mitochondrial morphology and function compared to RIF-1 parent cells may be responsible for the resistance of RIF-8A cells to Ph-PDT (Sharkey et al. 1993). However since Ph-PDT, UV, and cisplatin have been shown to induce DNA damage, other cellular factors such as altered DNA repair capabilities may play a role in the survival of RIF-8A cells following treatment with these damaging agents.

HCR assesses the ability of nontreated host cells to repair and replicate viral DNA that has been damaged by an agent such as gamma radiation (Parsons et al. 1986, Eady et al. 1992), UV (Ryan et al. 1986, Arnold and Rainbow 1996, Rainbow 1989) or chemicals such as cisplatin (Maynard et al. 1989, Zeng Rong et al. 1995, Jennerwein et al. 1991). HCR has been performed in many types of mammalian cells including human fibroblasts (Day et al. 1975, Ryan et al. 1986, Rainbow 1980), HeLa cells (Eady

et al. 1992) chinese hamster ovary cells (Arnold and Rainbow 1996) and murine leukemia cells (Jennerwein et al. 1991). HCR has been used to assess DNA repair capabilities in cells from patients with several genetic instability syndromes including ataxia telangiectasia (Hilgers et al. 1989, Bennett and Rainbow 1988), Fanconi's anemia (Nocentini 1992) xeroderma pigmentosum (Day et al. 1975, McKay and Rainbow 1996, Rainbow et al. 1981, Maynard et al. 1989, Nocentini et al. 1992), and Cockayne's syndrome (McKay and Rainbow 1996, Rainbow 1989).

Reduction in HCR of viral DNA synthesis has been seen in UV sensitive mouse cell lines (Arnold and Rainbow 1996) as well as UV sensitive xeroderma pigmentosum human cell lines (Rainbow 1989). RIF-1 cells have shown increased sensitivity to UV compared to RIF-8A cells (Di Prospero et al. 1996, Roy et al. 1996). It was therefore of interest to measure the HCR of a UV-damaged virus in RIF-1 and RIF-8A cells. In the following work, the HCR of a UV-damaged adenovirus based recombinant AdCA35*lacZ* carrying the *lacZ* reporter gene was examined in RIF-1 and RIF-8A cells. The *lacZ* reporter gene product (β-gal) is easily detected through biochemical means. AdCA35*lacZ* is a recombinant human adenovirus (Ad) virus which has been used as a vector to carry the *lacZ* reporter gene under the control of the murine cytomegalovirus (MCMV) immediate early promoter, inserted into the E1 deleted region of the Ad genome (Graham and Prevec, 1991). The repair of a UV damaged reporter gene measured by the production of reporter gene product reflects the ability of host cells to repair UV damage in an actively transcribed gene.

Mammalian cells have been shown to respond to pretreatment with subtoxic doses of UV light, ionizing radiation or various chemical DNA damaging agents with increased virus reactivation and mutagenic DNA repair, much like the SOS response seen in bacteria (Dion and Hamelin 1987, Hilgers et al. 1989, Coppey and Menezes 1981). In mammalian cells, this phenomenon has been referred to as enhanced reactivation. ER of UV damaged virus in mammalian cells is suggested to result from an inducible DNA repair pathway (Rainbow 1981). In normal human fibroblasts, ER has been seen following UV pretreatment (Hilgers et al. 1989, Coppey and Menezes 1981, Dion and Hamelin 1987, Bennett and Rainbow 1987, Abrahams et al. 1988), and heat shock pretreatment (McKay and Rainbow 1996). In rat hepatocytes, pretreatment of cells with UV resulted in ER of UV irradiated Herpes Simplex virus type 1 (HSV-1) (Zurlo and Yager 1984). ER has also been reported in CV-1 African green monkey cells following both UV (Brown and Cerutti 1989) and hyperthermic pretreatments (Lytle and Carney 1988). In this work, ER of a UV-damaged reporter gene and enhanced expression of β-gal activity from a non-damaged reporter gene have been examined in RIF-1 and RIF-8A cells following PDT, UV, cisplatin, or hyperthermia pretreatments.

Materials and Methods

Cells

RIF-1 cells were provided by Dr. Barbara Henderson of Roswell Park Memorial Institute, Buffalo, New York. RIF-8A cells were obtained from Dr. Gurmit Singh of the Hamilton Regional Cancer Clinic and McMaster University, Dept. of Pathology, Hamilton, ON. RIF-1 and RIF-8A cells were grown in monolayer and maintained in Eagles α-minimal essential media (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (100 μg/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B; Gibco BRL) (growth media), and incubated at 37°C (5% CO_2/95% air in a humid environment).

Virus

The AdCA35*lacZ* is a nonreplicating Ad5 derived virus expressing *lacZ* under control of the MCMV immediate early promoter. This construct expresses β-gal in murine cells without replication of the virus (Morsy et al. 1993). Virus was obtained from Christina Addison and Dr. Frank Graham, Dept. of Biology, McMaster University, Hamilton, ON (Graham and Prevec 1991). Virus stock was frozen at -20°C in a filter sterilized solution of 10% glycerol in non-supplemented α-MEM.

Host Cell Reactivation Assay

Preparation of Cells

Cells were seeded at a density of 4 X 10^4 cells per well in 96-well microtitre plates in growth media. Plates were incubated at 37°C (5% CO_2/95% air in a humid environment) for 24 hours to allow cells to adhere to the bottom surface of the well prior to infection with virus.

UV-irradiation of Virus

UV irradiation of virus has previously been reported (Bennett and Rainbow, 1988). Viral suspensions in 1.6 mL α-MEM (1% antibiotic-antimycotic solution) were irradiated in 35 mm dishes on ice with continuous stirring. Virus was irradiated using a General Electric germicidal lamp (model G8T5) emitting predominantly at 254 nm. Viral particles were UV irradiated with 50 J/m^2 - 500 J/m^2 at an incident fluence rate of 2J/m^2/sec (J-255 shortwave UV meter, Ultraviolet Products, San Gabriel, CA). Aliquots of 100 μL were removed for each exposure to the virus and diluted in α-MEM (1% antibiotic-antimycotic). Cells were infected with virus at a multiplicity of infection (MOI) of 2 plaque forming units (pfu)/cell using 40 μL of viral suspension.

Measuring β-gal Activity

After the 24 hour incubation period, cells were assayed for β-gal activity by addition of 50 μL of Solution 1 (1mM PMSF, 0.5%NP-40 in 250mM Tris HCl at pH 7.8), 100 μL of the reaction buffer Solution 2 (10mM KCl, 1mM $MgSO_4$, 100mM sodium phosphate and 50mM β-mercaptoethanol all adjusted to pH 7.5), and 75 μL of Solution 3 (4g/L O-nitrophenol b-D-galactopyranoside (ONGP) in 100mM sodium phosphate at pH 7.5). Plates were incubated for a period of 20 minutes after the addition of Solution 1, 10 minutes following the addition of Solution 2, and 2 hours following addition of Solution 3. β-gal activity was read in all plates at OD_{405} using a spectrophotometer.

β-Gal Activity Time Course Experiments

The incubation time required following addition of Solution 3 to RIF-1 and RIF-8A cells, in order to obtain appropriate OD_{405} measurements of β-gal activity, was examined. This was done by concurrently infecting a series of plates seeded with RIF-1 or RIF-8A cells and waiting between 1-48 hours prior to OD_{405} measurement of β-gal activity, over a period of 48 hours.

Enhanced Reactivation Assays

The procedure for this assay was identical to the HCR assay, except that cells were pretreated with either Ph-PDT, JM2929-PDT, UV, cisplatin, or hyperthermia

immediately before virus infection.

PDT pretreatment

Photofrin was obtained from Quadralogic Technologies Inc., Vancouver, B.C. JM2929 was obtained from Johnson Matthey Inc., PA,. Cells were seeded and incubated for 24 hours as described in the HCR assay. Growth media was then aspirated and replaced with fresh growth media containing either Photofrin or JM2929 at concentrations ranging from 5-20 μg/mL. Plates were then incubated for 24 hours after which time media was aspirated and replaced with fresh growth media without photosensitizer. Plates were placed on a 100 cm X 50 cm light diffusing surface illuminated by a bank of fluorescent tubes (Philips type TL/83) filtered with red acetate filters (Roscolux, No.19, Rosco, CA) to give wide band illumination above 585 nm. The energy fluence rate was approximately 9.2 $J/m^2/sec$. The time of illumination was 5 minutes for each plate which resulted in an incident energy fluence of approximately 2.76×10^3 J/m^2. Media was removed from each well once more before viral suspension was added. The remainder of the assay followed the procedure outlined in the HCR protocol. All procedures involving photosensitizer were carried out under conditions of minimal ambient lighting.

UV pretreatment

Cells were seeded as described in the HCR protocol. After 24 hours incubation, media was aspirated from each well and cells were overlaid with 40 μL of phosphate

buffer saline (PBS) (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4). Cells were then UV irradiated with 2-20 J/m^2 at an incident fluence rate of $1J/m^2/sec$ (J-255 shortwave UV meter, Ultraviolet Products, San Gabriel, CA) using a General Electric germicidal lamp (model G8T5) emitting predominantly at 254 nm.

Hyperthermia Pretreatment

Cells were seeded as described in the HCR protocol. Prewarmed growth media at 43.5°C ± 0.25 was added to each well before sealing the 96-well plate with PVC tape. This procedure took no longer than 1 minute. Plates were then submerged into a water bath whose temperature was maintained at 43.5°C ± 0.25°C, for time periods ranging from 15-30 minutes. Immediately following hyperthermia treatment, cells were infected with virus.

Cisplatin Pretreatment

Cells were seeded as described in the HCR protocol. Media from each well was removed prior to adding growth media containing 12.5 μM cisplatin. Cells were treated with cisplatin for 1 hour prior to removal and addition of virus.

Colony Forming Assay

Colony forming assays (CFAs) were conducted on RIF-1 and RIF-8A cells to examine their survival following hyperthermia treatments of 42°C, 43.5°C, and 46°C ± 0.25°C. All CFAs were performed in 6 well plastic petri dishes (Falcon, Lincoln Park,

NJ). Appropriate numbers of cells were seeded in order to count between 100-200 colonies per well post treatment. Following a six hour attachment period, growth media was aspirated from the wells and replaced with fresh prewarmed growth media. Plates were then sealed with PVC tape before submersion into a prewarmed water bath for time periods between 15-60 minutes. Plates were incubated at 37°C (5% CO_2/95% air in a humid environment) for 4 days. Growth media was then aspirated and wells were rinsed with 1mL PBS. Cells were stained with methylene blue (5% w:v , 70% methanol and 30% dH_2O). The stain was allowed to dry for 24 hours before colonies greater than 20 cells were counted.

RESULTS

Time Course of β-gal expression following infection of RIF cells with AdCA35lacZ

The HCR of β-gal expression at different time points following infection of cells by AdCA35*lacZ* in RIF-1 and RIF-8A cells was examined. Typical results obtained from a single experiment (Figure 1) indicate similar time courses of β-gal expression in both RIF-1 (Figure 1A) and RIF-8A (Figure 1B) cells. A twenty-four hour incubation period post virus infection, followed by a 4 hour incubation after the addition of Solution 3, was used for conducting the HCR and ER assays in the present work.

Host Cell Reactivation Assays

RIF cells were infected with non-irradiated and UV-irradiated AdCA35*lacZ*. Representative survival curves for β-gal expression of UV irradiated Ad5CA35*lacZ* in RIF-1 and RIF-8A cells are shown in Figure 2. It can be seen that there is a similar UV exposure dependent decrease in relative β-gal activity following infection of either RIF-1 and RIF-8A cells. Survival points for each experiment were fitted to the linear quadratic equation ($SF=e^{-(\alpha x+\beta x^2)}$) (where SF is the surviving fraction of β-gal activity). D_{37} values (dose required to reduce the fraction of survival cells to 37%) were extrapolated for 15 independent experiments. The relative D_{37} value (D_{37} RIF-1cells/ D_{37} RIF-8A cells) was determined for each experiment. The mean relative D_{37} ± standard error of 15 independent experiments was 1.267 ± 0.10049 ($p<0.01$),

indicating a small, but significant increase in the HCR of a UV-damaged reporter gene in RIF-1 cells compared to RIF-8A cells.

Enhanced Reactivation Assays

In the present work, we examined the ER of a UV damaged reporter gene in RIF-1 and RIF-8A cells following pretreatment with Ph-PDT, JM-PDT, UV, cisplatin, and hyperthermia. For each treatment dose to cells, β-gal activity from UV damaged AdCA35*lacZ* was compared to β-gal activity from non-damaged AdCA35*lacZ*. Typical results from a single experiment are shown for one dose or exposure to cells (Figures 3,6,9,12,14), as well as one UV exposure to virus (300 J/m^2) (Figures 4,7,10,15), for each type of damaging agent given to RIF-1 and RIF-8A cells. The expression of β-gal activity from a non-damaged reporter gene was also examined for RIF-1 and RIF-8A cells following each dose or exposure of damaging agent to cells (Figures 5,8,11,16).

Results for colony survival, ER of a UV damaged reporter gene, and enhanced expression of β-gal activity from a non-damaged reporter gene in RIF-1 and RIF-8A cells, following treatment with each damaging agent, are summarized in Table I.

Ph-PDT Enhanced Reactivation

RIF-8A cells have previously been reported to have increased clonogenic survival following Ph-PDT compared to RIF-1 parent cells (Singh et al. 1991) Enhanced reactivation of a UV damaged reporter gene was examined in RIF-1 and RIF-

8A cells pretreated with Ph-PDT. Each individual experiment showed enhanced reactivation of a UV damaged reporter gene in Ph-PDT treated RIF-1 cells, but not in Ph-PDT treated RIF-8A cells, over the range of doses examined (Refer to Table I). Figure 3 shows typical results for enhanced reactivation of a UV damaged reporter gene in 15 μg/mL Ph-PDT treated RIF-1 (Figure 3A) and RIF-8A (Figure 3B) cells, compared to nontreated cells. Figure 4 shows pooled results (from two experiments) of the ratio of β-gal activity (treated cells/non-treated cells) from reporter gene irradiated with 300 J/m^2 UV in RIF-1 and RIF-8A cells, pretreated with Ph-PDT.

Enhanced expression of β-gal activity from an undamaged reporter gene was also examined in Ph-PDT treated RIF-1 and RIF-8A cells. In each individual experiment RIF-8A cells, but not RIF-1 cells showed enhanced expression of β-gal activity from an undamaged reporter gene following Ph-PDT over the range of doses examined (Refer to Table I). Figure 5 shows pooled results (for two experiments) of the relative β-gal activity (treated cells/non-treated cells) from a non-damaged reporter gene in RIF-1 and RIF-8A cells following Ph-PDT pretreatment.

JM2929-PDT Enhanced Reactivation

RIF-1 and RIF-8A cells have been reported to have similar survival following JM2929-PDT (Roy et al. 1996). Enhanced reactivation of a UV damaged reporter gene was examined in RIF-1 and RIF-8A cells pretreated with JM2929-PDT. Each individual experiment showed increased reactivation of a UV damaged reporter gene in JM2929-PDT treated RIF-1 cells, compared to JM2929-PDT treated RIF-8A cells, over the range

of doses examined (Refer to Table I). Figure 6 shows typical results for enhanced reactivation of a UV damaged reporter gene in 15 μg/mL JM2929-PDT treated RIF-1 (Figure 6A) and RIF-8A (Figure 6B) cells, compared to nontreated cells. Figure 7 shows pooled results (from two experiments) of the ratio of β-gal activity (treated cells/non-treated cells) from reporter gene irradiated with 300 J/m^2 UV in RIF-1 and RIF-8A cells pretreated with JM2929-PDT.

Enhanced expression of β-gal activity from an undamaged reporter gene was also examined in JM2929-PDT treated RIF-1 and RIF-8A cells. In each individual experiment, a similar level of decreased expression of β-gal activity from an undamaged reporter gene was observed for RIF-1 and RIF-8A cells, over the range of JM2929-PDT doses examined (Refer to Table I). Figure 8 shows pooled results (for two experiments) of the relative β-gal activity (treated cells/non-treated cells) from a non-damaged reporter gene in RIF-1 and RIF-8A cells following JM2929-PDT pretreatment.

UV Enhanced Reactivation

RIF-8A cells have previously been shown to have increased resistance to UV compared to parent RIF-1 cells (Di Prospero et al. 1996, Roy et al. 1996). Enhanced reactivation of a UV damaged reporter gene was examined in RIF-1 and RIF-8A cells pretreated with UV irradiation. Each individual experiment showed increased reactivation of a UV damaged reporter gene in UV treated RIF-1 cells, compared to UV treated RIF-8A cells, at a UV exposure of 5J/m^2 (Refer to Table I). Figure 9 shows typical results for enhanced reactivation of a UV damaged reporter gene in 5J/m^2 UV-

irradiated RIF-1 (Figure 9A) and RIF-8A (Figure 9B) cells, compared to nontreated cells. Figure 10 shows pooled results (from three experiments) of the ratio of β-gal activity (treated cells/non-treated cells) from reporter gene irradiated with 300 J/m^2 UV in RIF-1 and RIF-8A cells pretreated with UV irradiation.

Enhanced expression of β-gal activity from an undamaged reporter gene was also examined in UV-irradiated RIF-1 and RIF-8A cells. In each individual experiment increased expression of β-gal activity from an undamaged reporter gene was seen for both RIF-1 and RIF-8A cells following UV irradiation over the range of exposures examined (Refer to Table I). Figure 11 shows pooled results (for three experiments) of the relative β-gal activity (treated cells/non-treated cells) from a non-damaged reporter gene in RIF-1 and RIF-8A cells following UV pretreatment.

Cisplatin Enhanced Reactivation

Ph-PDT resistant RIF-8A cells have previously been shown to have a cross resistance to cisplatin (Moorehead et al. 1994). Enhanced reactivation of a UV damaged reporter gene was examined in RIF-1 and RIF-8A cells pretreated with 12.5 μM cisplatin. Each individual experiment showed no difference in reactivation of a UV damaged reporter gene between cisplatin treated and non treated RIF-1, as well as RIF-8A cells, for the cisplatin dose examined (Refer to Table I). Figure 12 shows typical results for enhanced reactivation of a UV damaged reporter gene in 12.5 μM cisplatin treated RIF-1 (Figure 12A) and RIF-8A (Figure 12B) cells, compared to nontreated cells.

Pooled results from three independent experiments also suggest no difference in expression of β-gal activity from an non-damaged reporter gene in cisplatin treated compared to non-treated cells, for both RIF-1 and RIF-8A cells (results not shown).

Colony Forming Assays of Heat Shock treated cells

Colony forming assays were performed for RIF-1 and RIF-8A cells over a dose range of 42°C to 46°C ± 0.25°C, for exposure periods ranging from 15-60 minutes. Each individual experiment showed no difference in sensitivity between RIF-1 and RIF-8A cells following hyperthermia treatment, over the range of doses examined. Pooled results from three independent experiments are shown in Figure 13.

Heat Shock Enhanced Reactivation

RIF-1 and RIF-8A cells appear to have similar survival following hyperthermia treatment, as was determined using the colony forming assay (Figure 12). Enhanced reactivation of a UV damaged reporter gene was examined in RIF-1 and RIF-8A cells pretreated with hyperthermia. Each individual experiment showed decreased reactivation of a UV damaged reporter gene in hyperthermia pretreated RIF-1 and RIF-8A cells, over the range of doses examined (Refer to Table I). Figure 14 shows typical results for enhanced reactivation of a UV damaged reporter gene in hyperthermia treated (30 min, 43.5°C ± 0.25) RIF-1 (Figure 14A) and RIF-8A (Figure 14B) cells, compared to nontreated cells. Figure 15 shows pooled results (from three experiments)

of the ratio of β-gal activity (treated cells/non-treated cells) from reporter gene irradiated with 300 J/m^2 UV in RIF-1 and RIF-8A cells pretreated with hyperthermia.

Enhanced expression of β-gal activity from an undamaged reporter gene was also examined in hyperthermia treated RIF-1 and RIF-8A cells. In each individual experiment decreased expression of β-gal activity from an undamaged reporter gene was seen for both RIF-1 and RIF-8A cells following hyperthermia pretreatment, over the range of doses examined (Refer to Table I). Figure 16 shows pooled results (for three experiments) of the relative β-gal activity (treated cells/non-treated cells) from a non-damaged reporter gene in RIF-1 and RIF-8A cells following UV pretreatment.

Results from each of the above assays are summarized in Table 1.

Figure 1: Time Course measurement of β-gal activity in RIF cells following infection with AdCA35*lacZ*.

RIF-1(A) and RIF-8A (B) cells were infected with AdCA35*lacZ* at an MOI of 2 pfu/cells. Measurements at OD_{405} of β-gal activity were conducted for 1hr (-■-), 5hr (-●-), 12hr (-▲-), 24 hr (-▼-) and 48 hr (-◆-) incubation periods following the 90 minute viral infection period. Shows results from one experiment conducted in triplicate trials. Each data point is the average ± standard error.

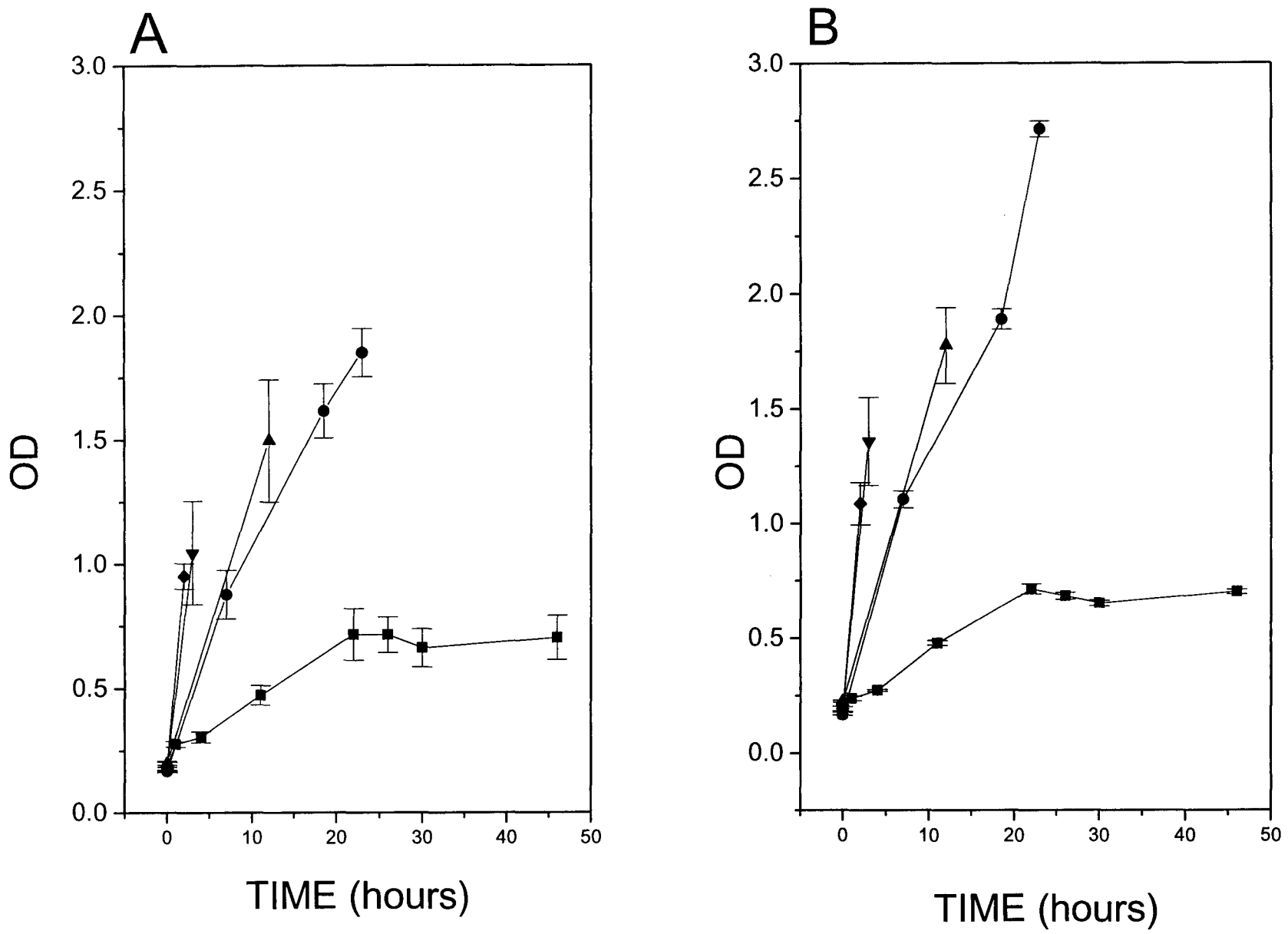
A
B
OD
TIME (hours)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50

Figure 2: Host Cell Reactivation of UV damaged AdCA35*lacZ* by RIF cells.

RIF-1 (-■-) and RIF-8A (-●-) cells were infected with non-irradiated and UV-irradiated AdCA35*lacZ*. Shows results from a typical experiment. Relative β-gal activity is the surviving fraction (SF) of reporter gene activity measured at OD_{405}. Each data point is the arithmetic mean ± standard error of three determinations from a single experiment.

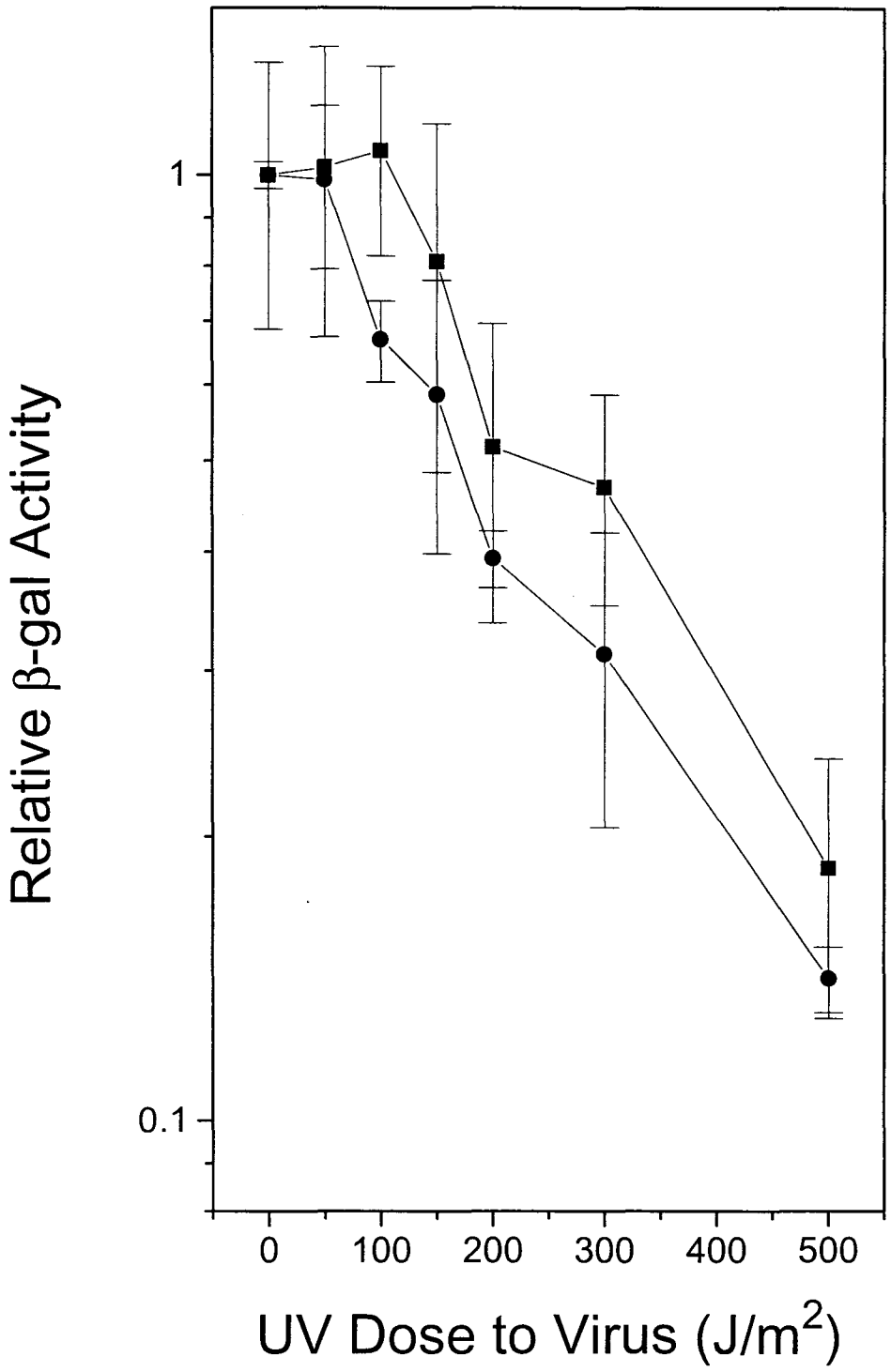
Relative β-gal Activity
1
0.1
0
100
200
300
400
500
UV Dose to Virus (J/m2)

Figure 3: Ph-PDT Enhanced reactivation of UV damaged reporter gene in RIF cells.

Shows typical results of β-gal activity from UV-irradiated AdCA35*lacZ* in untreated (-■-) and 15 μg/mL Ph-PDT treated (-●-) RIF-1 (A) and RIF-8A (B) cells. Relative β-gal activity is the surviving fraction (SF) of reporter gene activity measured at OD_{405}. Each data point is the arithmetic mean ± standard error of three determinations from a single experiment.

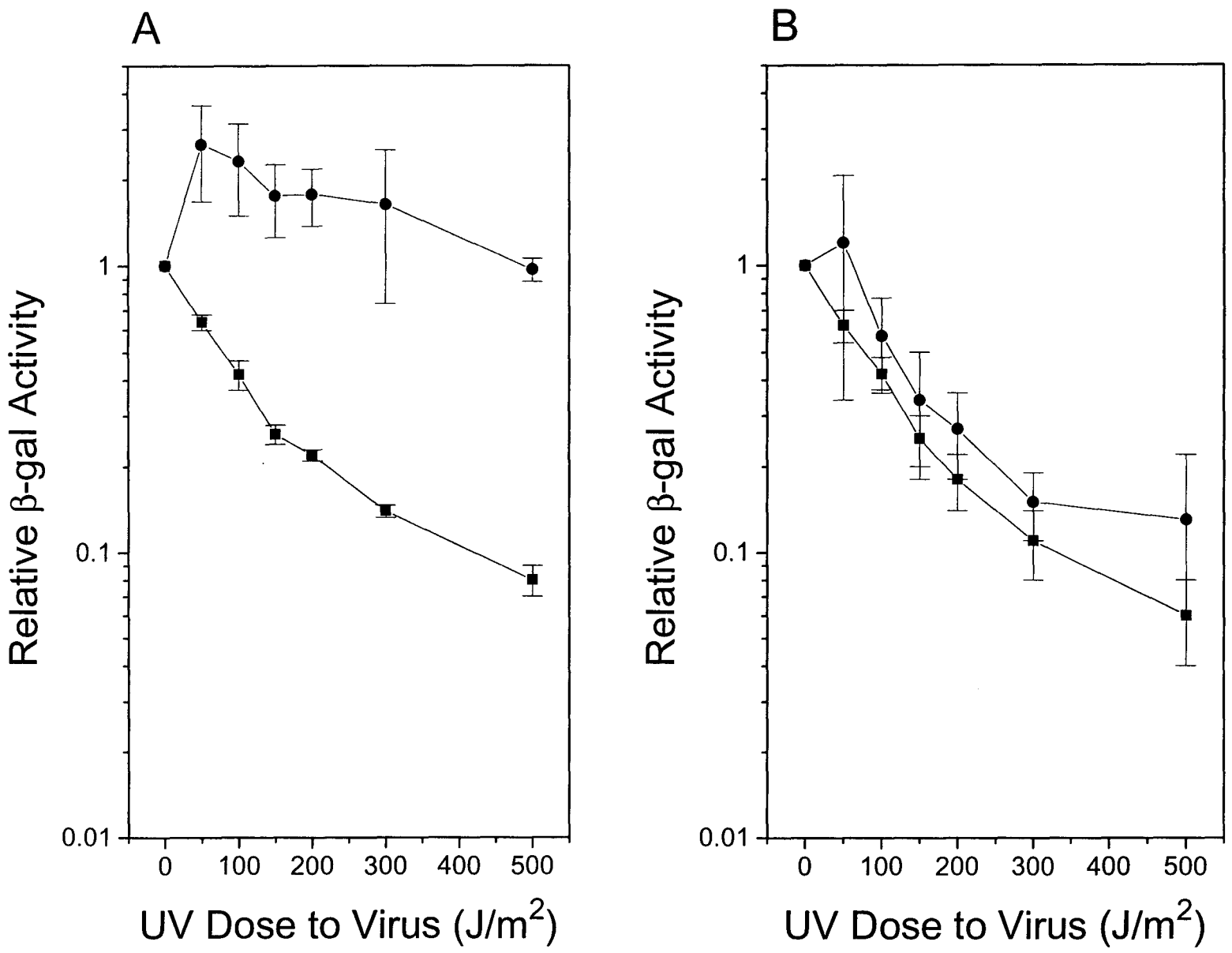
A
B
Relative β-gal Activity
1
0.1
0.01
0
100
200
300
400
500
UV Dose to Virus (J/m2)

Figure 4: Enhanced reactivation of AdCA35*lacZ* irradiated with 300 J/m^2 UV light by Ph-PDT treated RIF cells

Ph-PDT treated RIF-1(-■-) and RIF-8A (-●-) cells were infected with UV irradiated and non-irradiated AdCA35*lacZ*. Shows pooled results of two experiments each conducted in triplicate. β-Gal activity was measured at OD_{405}. Relative β-gal activity of 300 J/m^2 UV light exposed AdCA35*lacZ* (pretreated cells/non-treated cells) was calculated. Each data point represents the arithmetic mean ± standard error.

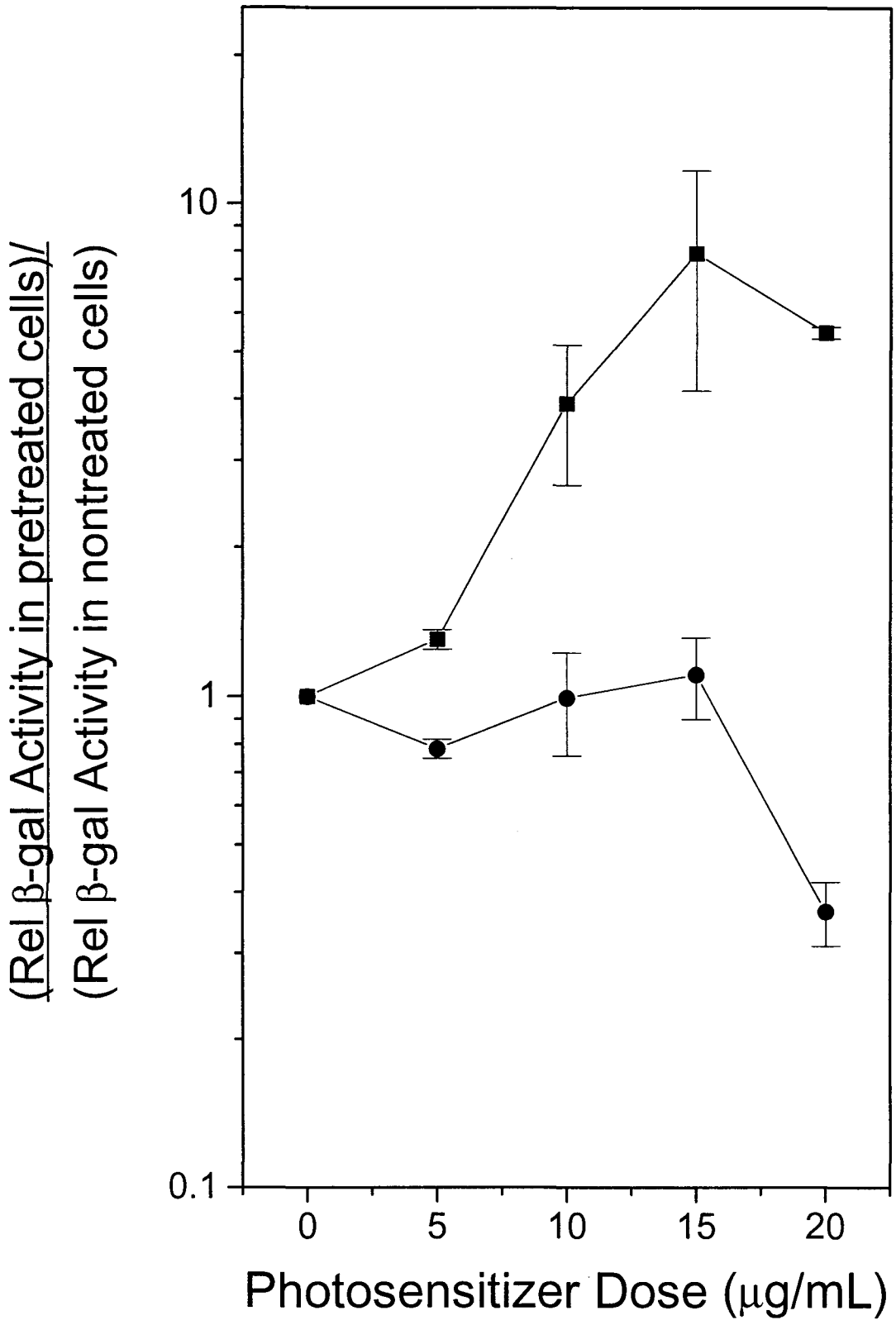
(Rel β-gal Activity in pretreated cells)/
(Rel β-gal Activity in nontreated cells)
10
1
0.1
0
5
10
15
20
Photosensitizer Dose (μg/mL)

Figure 5: Enhanced expression of β-gal activity in Ph-PDT treated RIF cells

Ph-PDT pretreated and untreated RIF-1 (-■-) and RIF-8A (-●-) cells were infected with non-irradiated AdCA35*lacZ*. Shows pooled results from two experiments each conducted in triplicate. β-Gal activity was measured at OD_{405}. Relative β-gal activity for undamaged reporter gene was calculated from the surviving fraction of reporter gene activity (treated cells/non-treated cells). Each data point represents the arithmetic mean ± standard error.

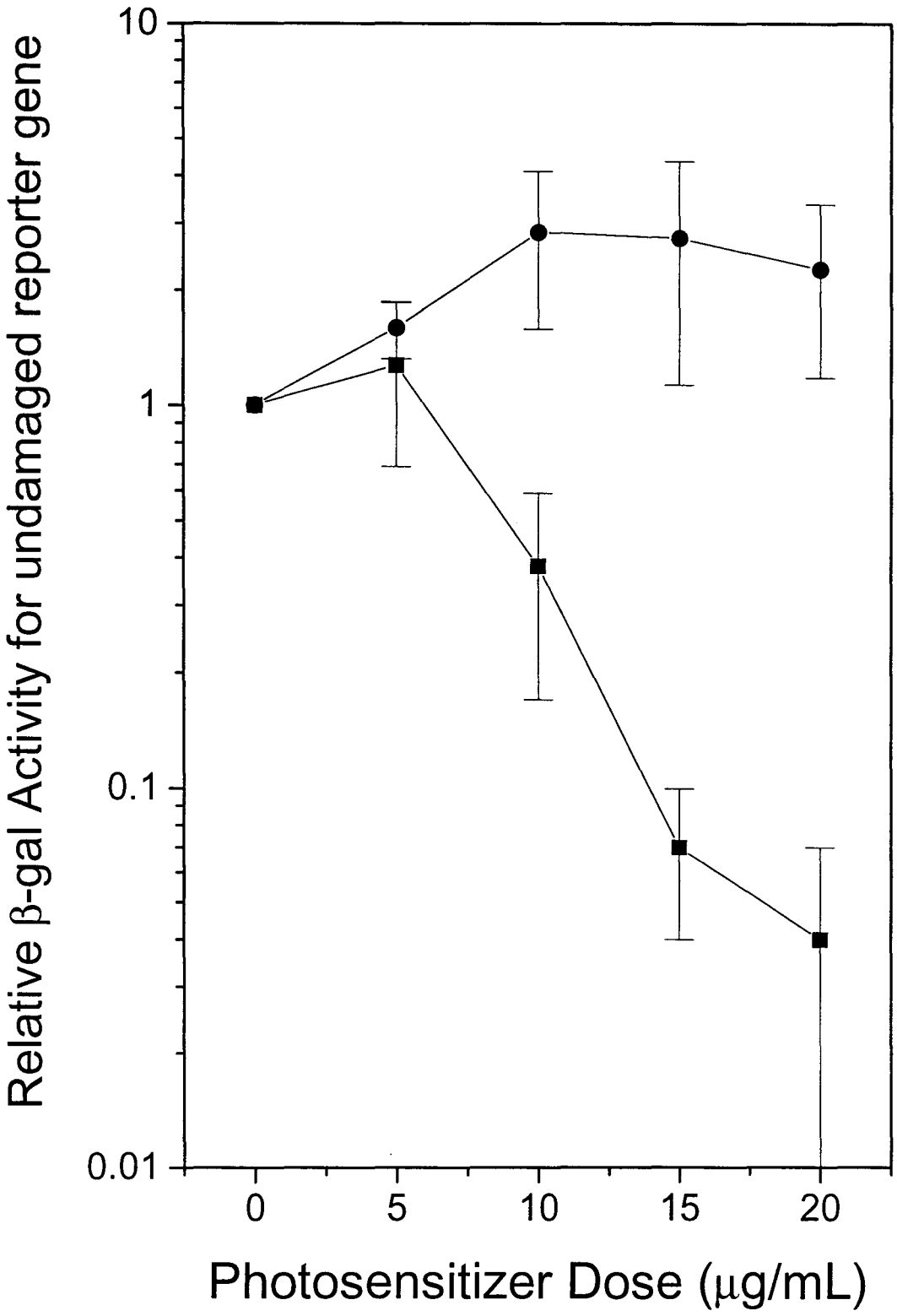
10
1
0.1
0.01
Relative β-gal Activity for undamaged reporter gene
0
5
10
15
20
Photosensitizer Dose (μg/mL)

Figure 6: JM2929-PDT Enhanced reactivation of UV damaged reporter gene in RIF cells.

Shows typical results of β-gal activity from UV-irradiated AdCA35*lacZ* in untreated (-■-) and 15 μg/mL JM-PDT treated (-●-) RIF-1 (A) and RIF-8A (B) cells. Relative β-gal activity is the surviving fraction (SF) of reporter gene activity measured at OD_{405}. Each data point is the arithmetic mean ± standard error of three determinations from a single experiment.

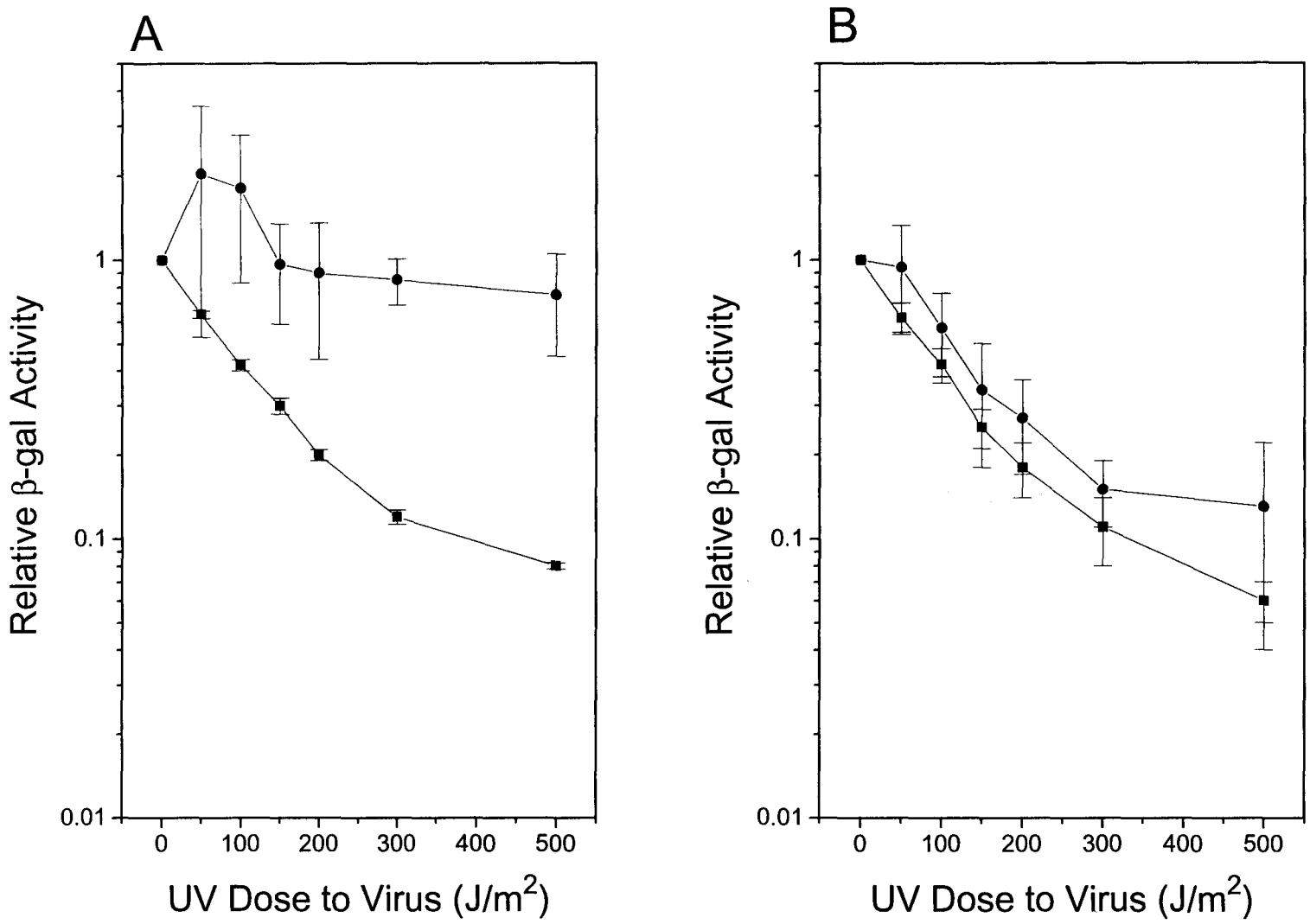
A
B
Relative β-gal Activity
1
0.1
0.01
0
100
200
300
400
500
UV Dose to Virus (J/m2)

Figure 7: Enhanced reactivation of AdCA35*lacZ* irradiated with 300 J/m^2 UV light by JM2929-PDT treated RIF cells

JM2929-PDT treated RIF-1(-■-) and RIF-8A (-●-) cells were infected with UV irradiated and non-irradiated AdCA35*lacZ*. Shows pooled results of two experiments each conducted in triplicate. β-Gal activity was measured at OD_{405}. Relative β-gal activity of 300 J/m^2 UV light exposed AdCA35*lacZ* (pretreated cells/non-treated cells) was calculated. Each data point represents the arithmetic mean ± standard error.

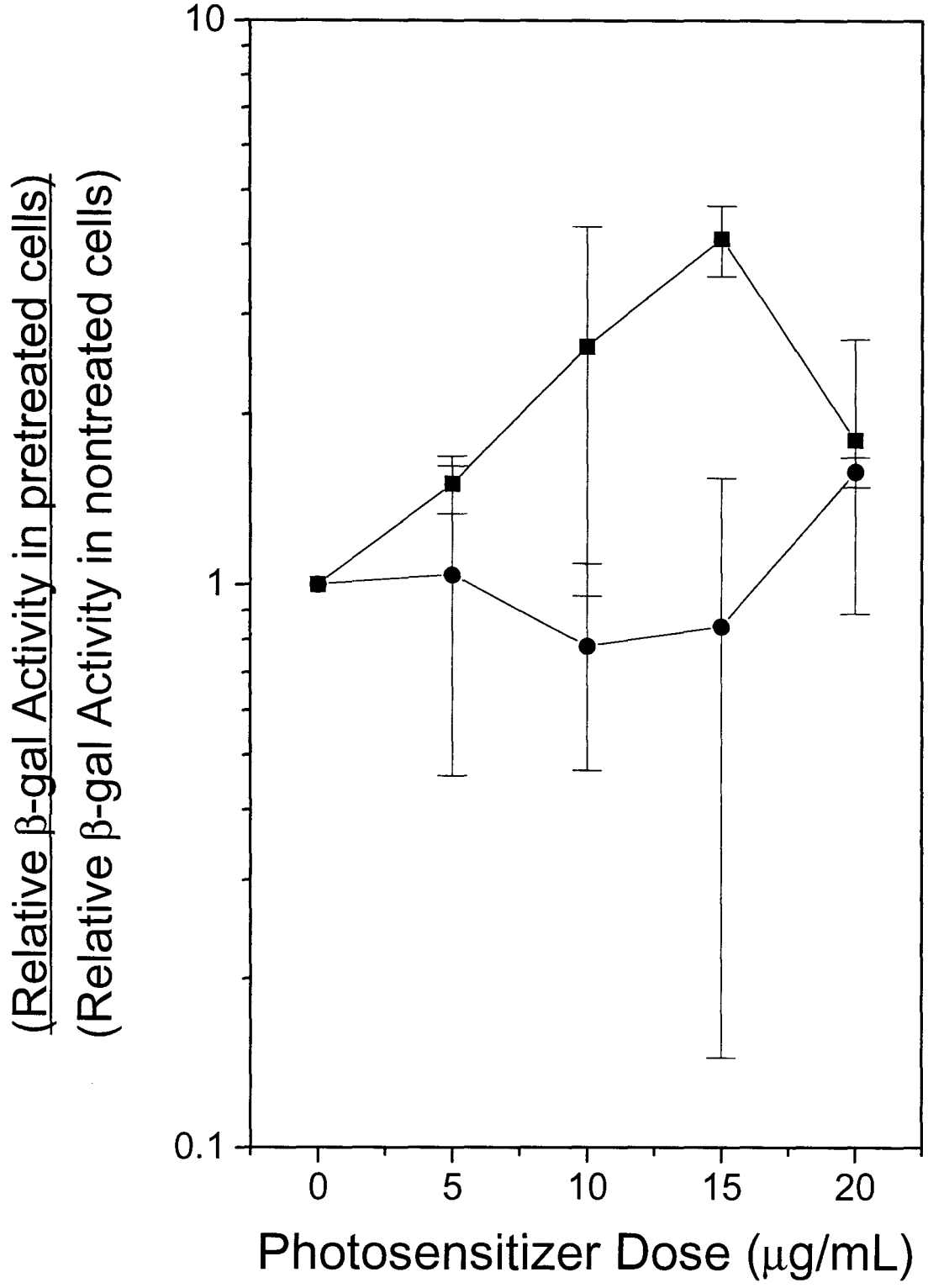
(Relative β-gal Activity in pretreated cells)
(Relative β-gal Activity in nontreated cells)
10
1
0.1
0
5
10
15
20
Photosensitizer Dose (μg/mL)

Figure 8: Enhanced expression of β-gal activity in JM2929-PDT treated RIF cells

JM2929-PDT pretreated and untreated RIF-1 (-■-) and RIF-8A (-●-) cells were infected with non-irradiated AdCA35*lacZ*. Shows pooled results from two experiments each conducted in triplicate. β-Gal activity was measured at OD_{405}. Relative β-gal activity for undamaged reporter gene was calculated from the surviving fraction of reporter gene activity (treated cells/non-treated cells). Each data point represents the arithmetic mean ± standard error.

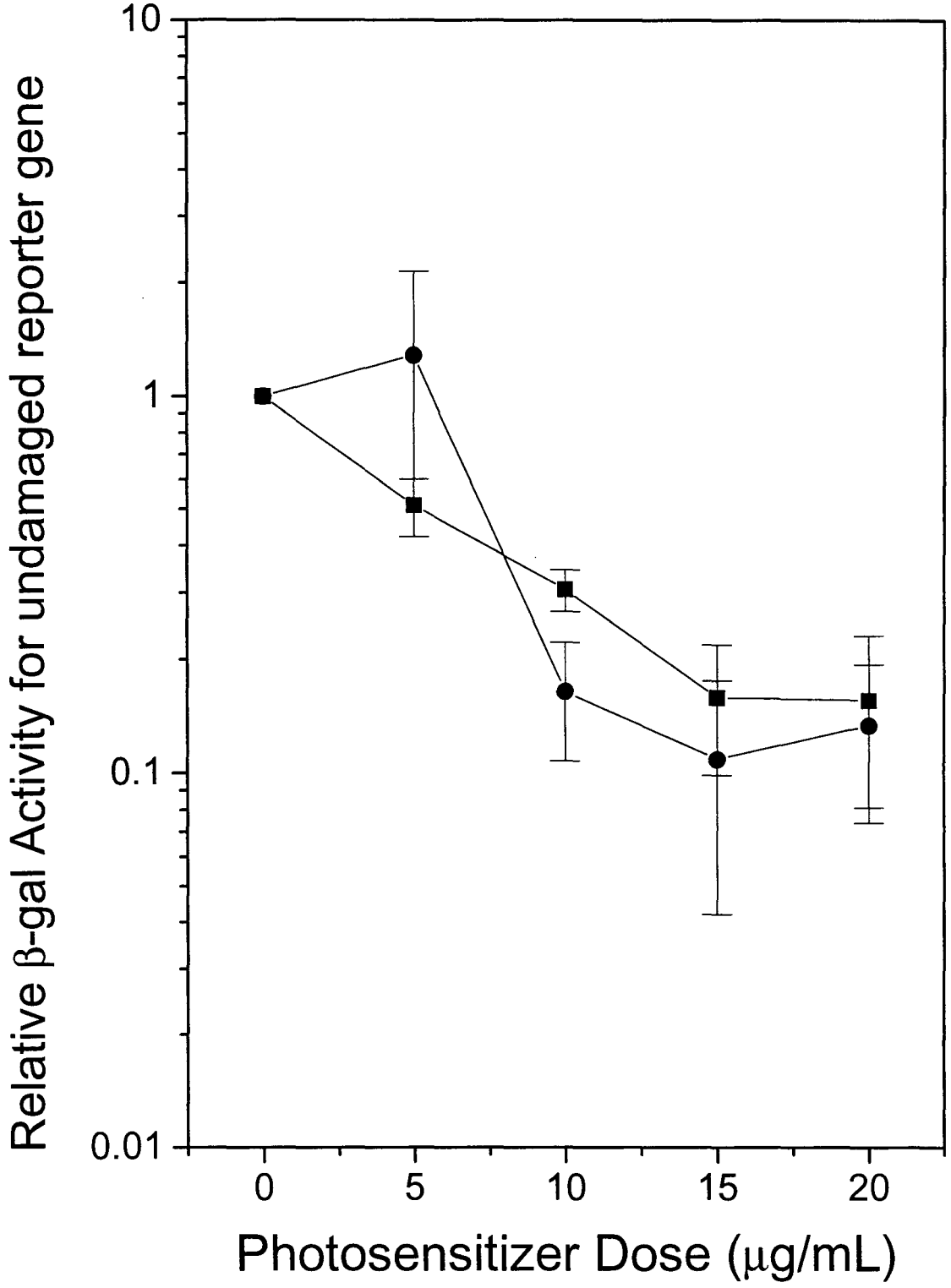
10
1
0.1
0.01
Relative β-gal Activity for undamaged reporter gene
0
5
10
15
20
Photosensitizer Dose (μg/mL)

Figure 9: UV Enhanced reactivation of UV damaged reporter gene in RIF cells.

Shows typical results of β-gal activity from UV-irradiated AdCA35*lacZ* in untreated (-■-) and 5 J/m^2 UV irradiation treated (-●-) RIF-1 (A) and RIF-8A (B) cells. Relative β-gal activity is the surviving fraction (SF) of reporter gene activity measured at OD_{405}. Each data point is the arithmetic mean ± standard error of three determinations from a single experiment.

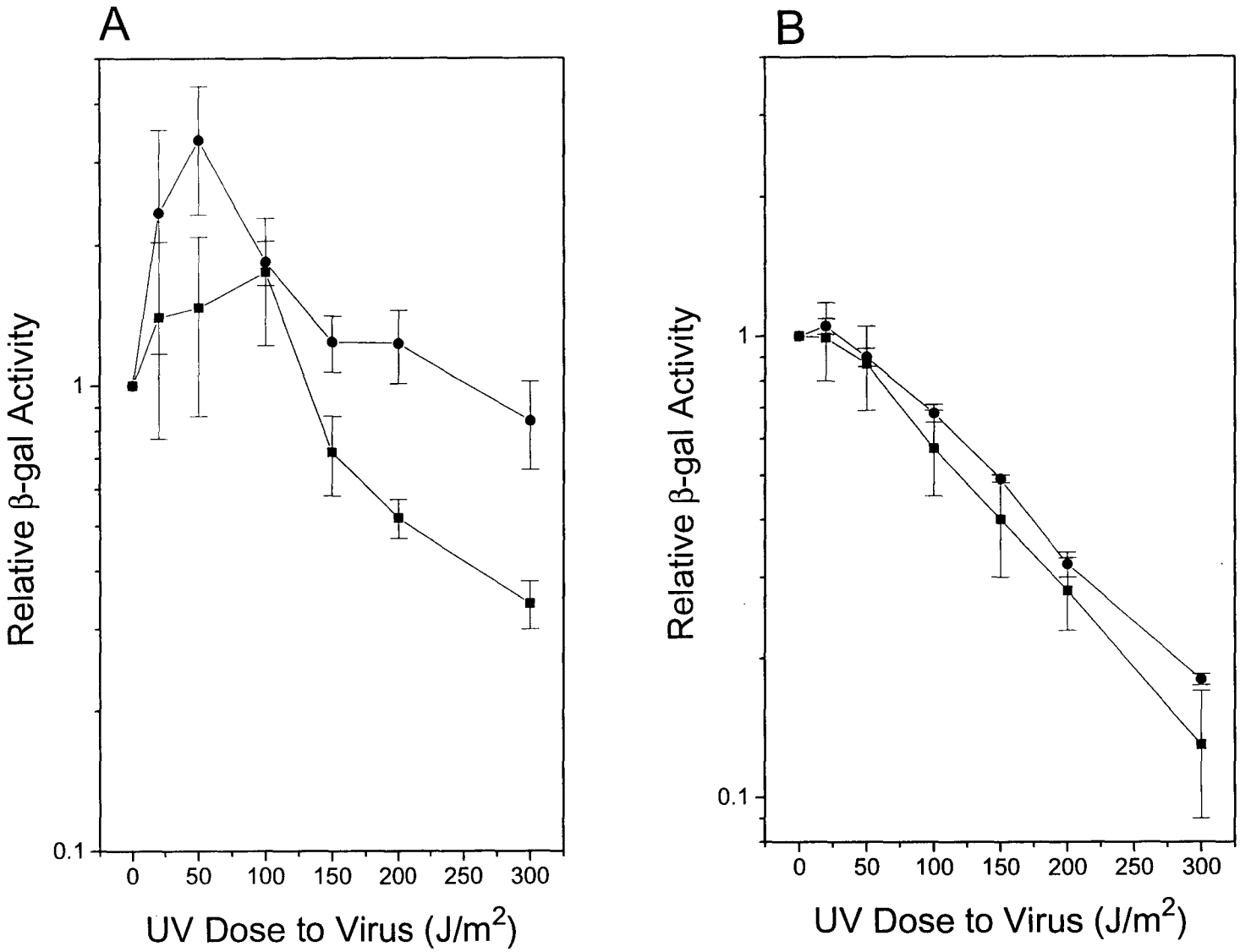
A
Relative β-gal Activity
1
0.1
0
50
100
150
200
250
300
UV Dose to Virus (J/m2)
B
Relative β-gal Activity
1
0.1
0
50
100
150
200
250
300
UV Dose to Virus (J/m2)

Figure 10: Enhanced reactivation of AdCA35*lacZ* irradiated with 300 J/m^2 UV light by UV treated RIF cells

UV treated RIF-1(-■-) and RIF-8A (-●-) cells were infected with UV irradiated and non-irradiated AdCA35*lacZ*. Shows pooled results of two experiments each conducted in triplicate. β-Gal activity was measured at OD_{405}. Relative β-gal activity of 300 J/m^2 UV light exposed AdCA35*lacZ* (pretreated cells/non-treated cells) was calculated. Each data point represents the arithmetic mean ± standard error.

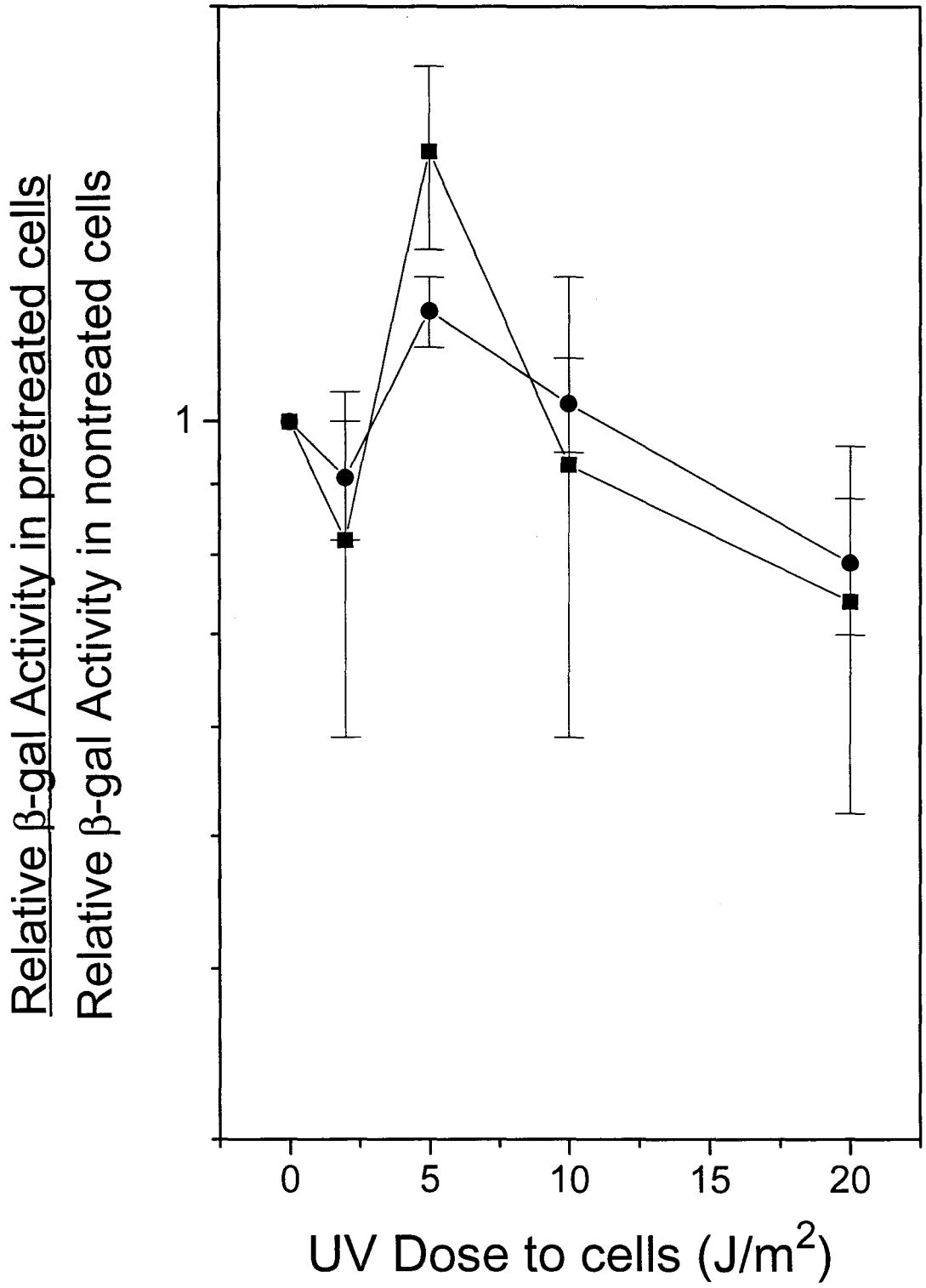
Relative β-gal Activity in pretreated cells
Relative β-gal Activity in nontreated cells
1
0
5
10
15
20
UV Dose to cells (J/m2)

Figure 11: Enhanced expression of β-gal activity in UV treated RIF cells

UV pretreated and untreated RIF-1 (-■-) and RIF-8A (-●-) cells were infected with non-irradiated AdCA35*lacZ*. Shows pooled results from two experiments each conducted in triplicate. β-Gal activity was measured at OD_{405}. Relative β-gal activity for undamaged reporter gene was calculated from the surviving fraction of reporter gene activity (treated cells/non-treated cells). Each data point represents the arithmetic mean ± standard error.

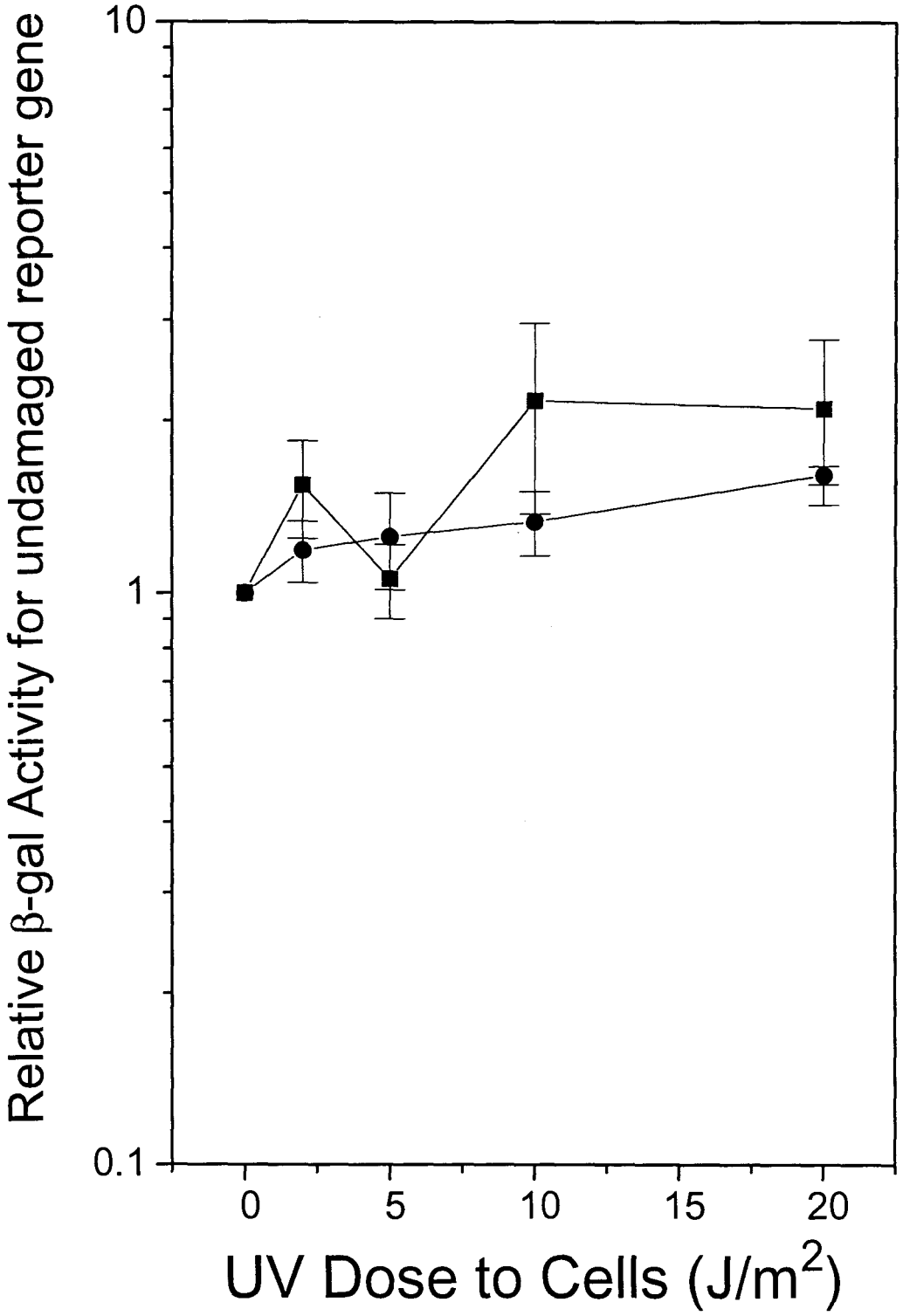
Relative β-gal Activity for undamaged reporter gene
10
1
0.1
0
5
10
15
20
UV Dose to Cells (J/m2)

Figure 12: Cisplatin Enhanced reactivation of UV damaged reporter gene in RIF cells.

Shows typical results of β-gal activity from UV-irradiated AdCA35*lacZ* in untreated (-■-) and 12.5 μM cisplatin treated (-●-) RIF-1 (A) and RIF-8A (B) cells. Relative β-gal activity is the surviving fraction (SF) of reporter gene activity measured at OD_{405}. Each data point is the arithmetic mean ± standard error of three determinations from a single experiment.

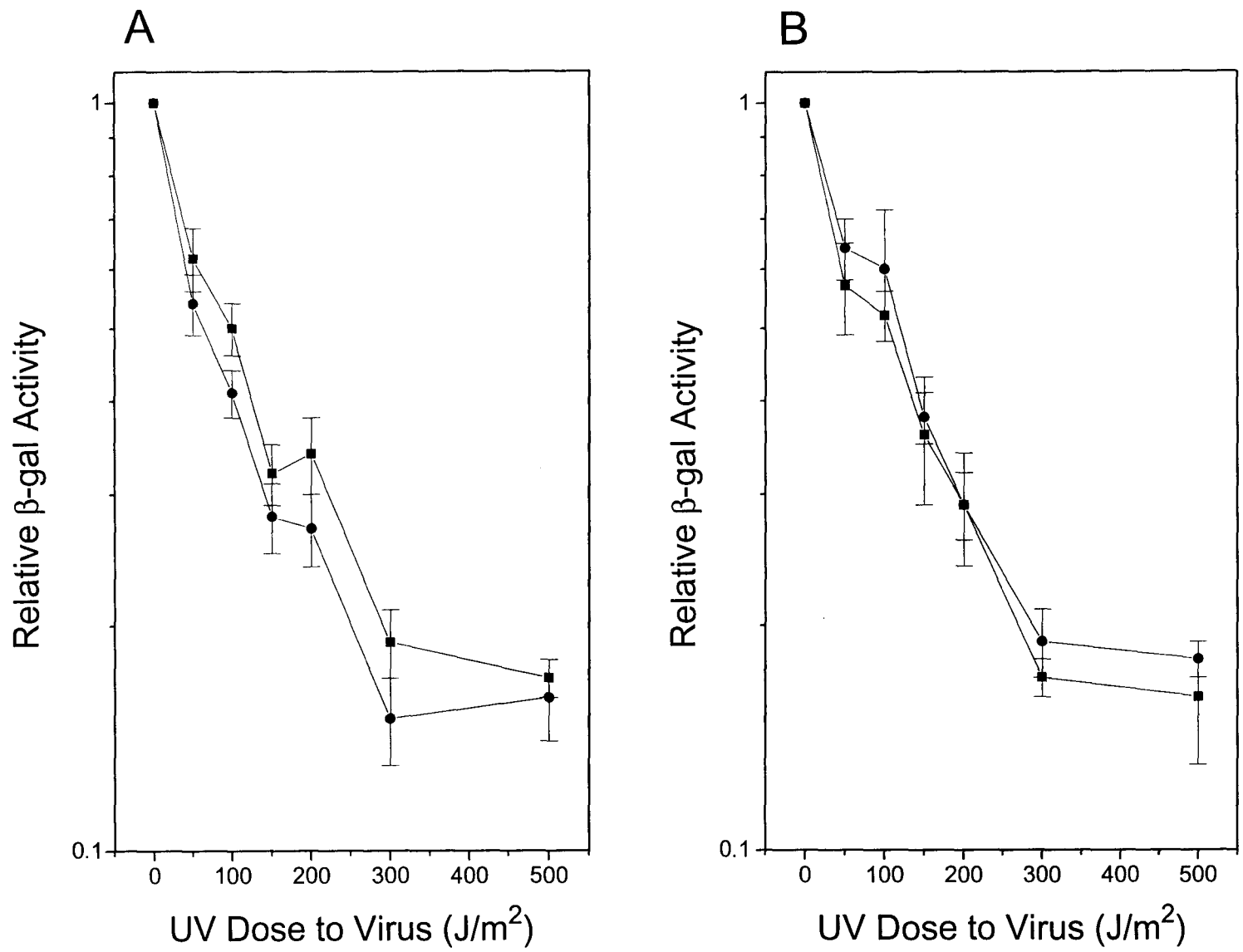
A
B
Relative β-gal Activity
1
0.1
0
100
200
300
400
500
UV Dose to Virus (J/m2)

Figure 13: Colony Forming Assay of Heat Shock treated RIF cells

Survival of RIF-1 cells exposed to 42°C (-■-), 43.5°C (-▲-), 46°C (-◆-) and RIF-8A cells exposed to 42°C (-●-), 43.5°C (-▼-) and 46°C (-✳-) was determined by colony forming assay. Shows pooled results of three experiments each conducted in triplicate. Each data point represents the arithmetic mean ± standard error.

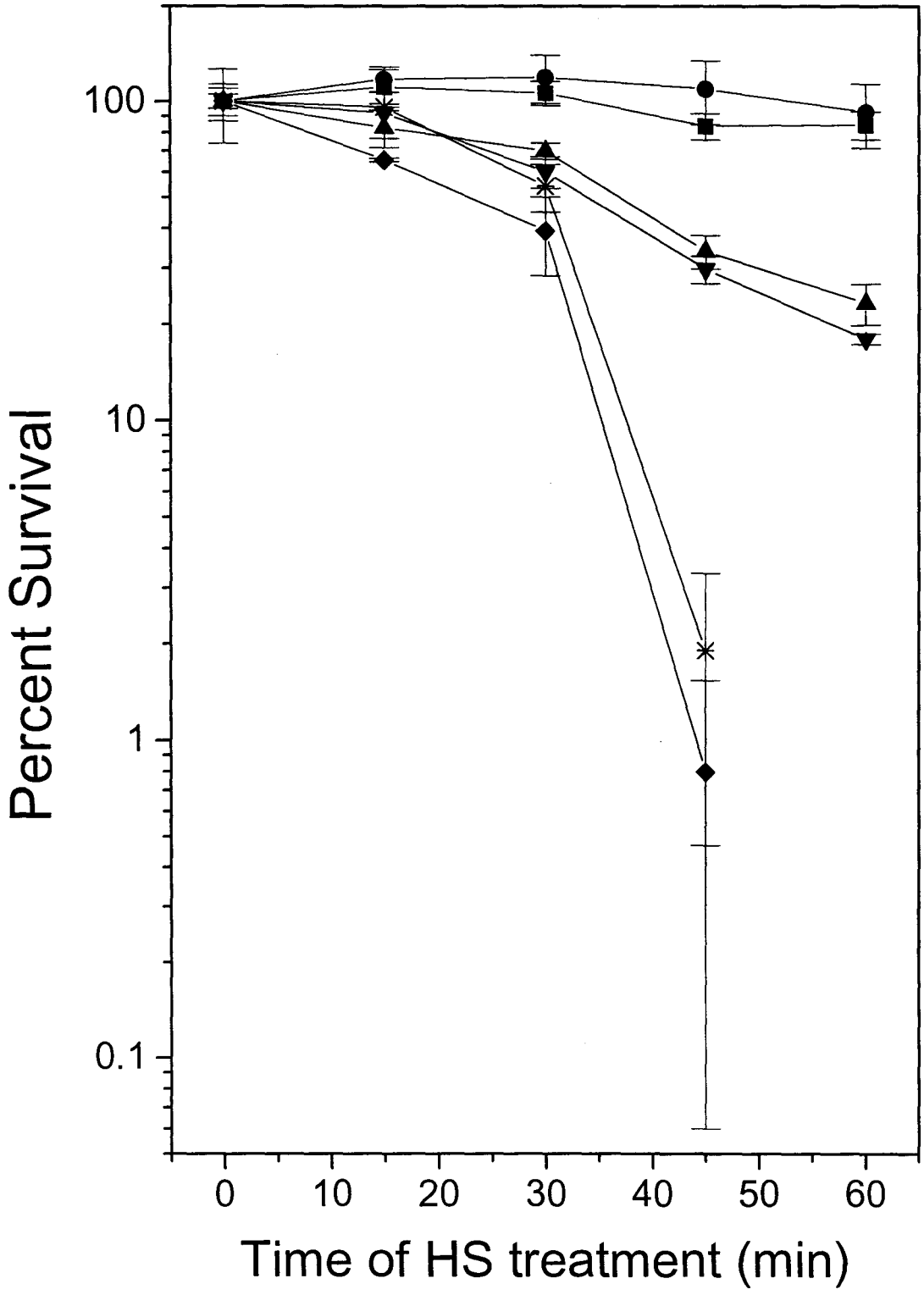
Percent Survival
100
10
1
0.1
0
10
20
30
40
50
60
Time of HS treatment (min)

Figure 14: Heat Shock Enhanced reactivation of UV damaged reporter gene in RIF cells.

Shows typical results of β-gal activity from UV-irradiated AdCA35*lacZ* in untreated (-■-) (30 min at 43.5°C ± 0.25) heat shock treated (-●-) RIF-1 (A) and RIF-8A (B) cells. Relative β-gal activity is the surviving fraction (SF) of reporter gene activity measured at OD_{405}. Each data point is the arithmetic mean ± standard error of three determinations from a single experiment.

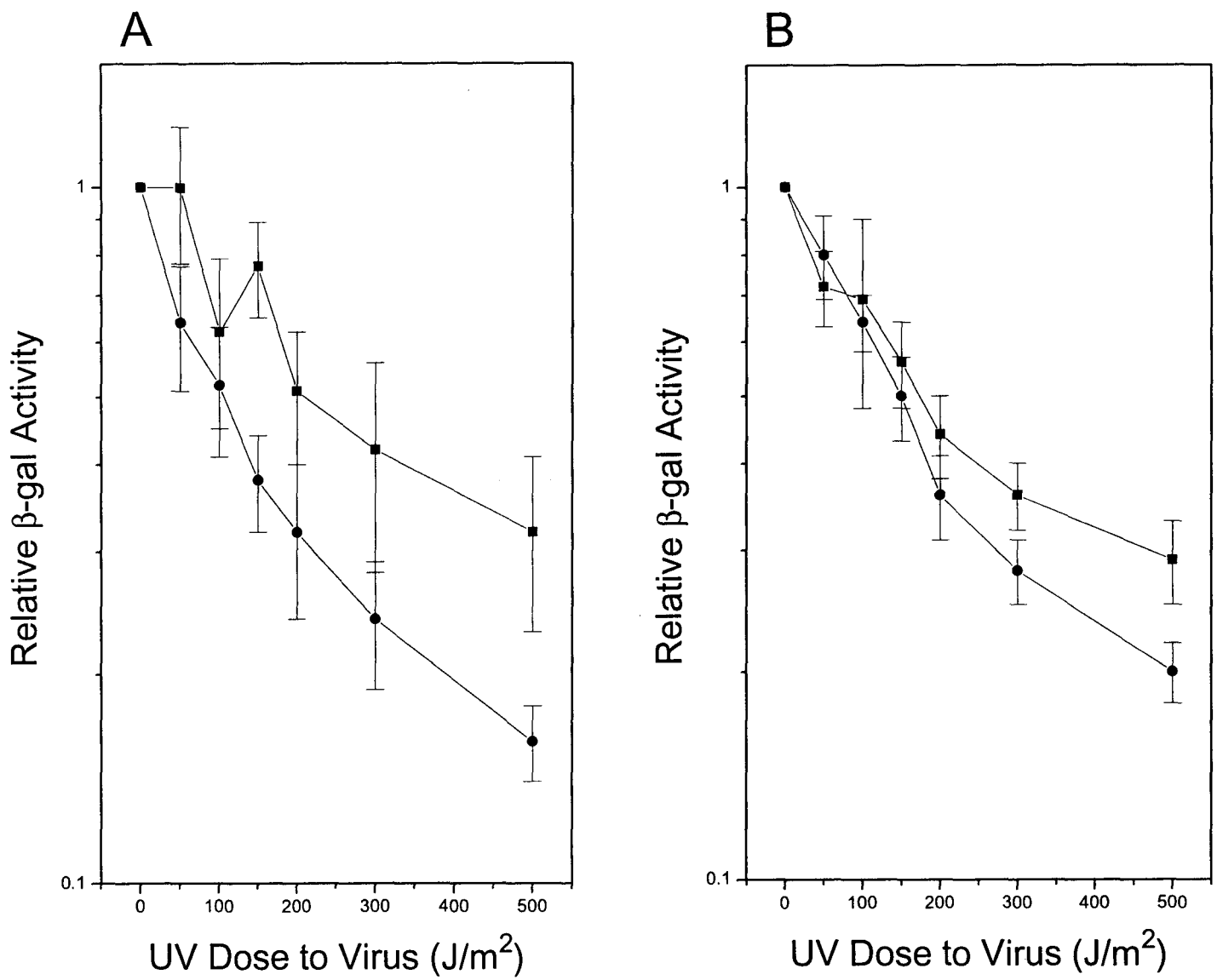
A
B
Relative β-gal Activity
1
0.1
0
100
200
300
400
500
UV Dose to Virus (J/m2)

Figure 15: Enhanced reactivation of AdCA35*lacZ* irradiated with 300 J/m^2 UV light by heat shock treated RIF cells

Heat shock treated RIF-1(-■-) and RIF-8A (-●-) cells were infected with UV irradiated and non-irradiated AdCA35*lacZ*. Shows pooled results of two experiments each conducted in triplicate. β-Gal activity was measured at OD_{405}. Relative β-gal activity of 300 J/m^2 UV light exposed AdCA35*lacZ* (pretreated cells/non-treated cells) was calculated. Each data point represents the arithmetic mean ± standard error.

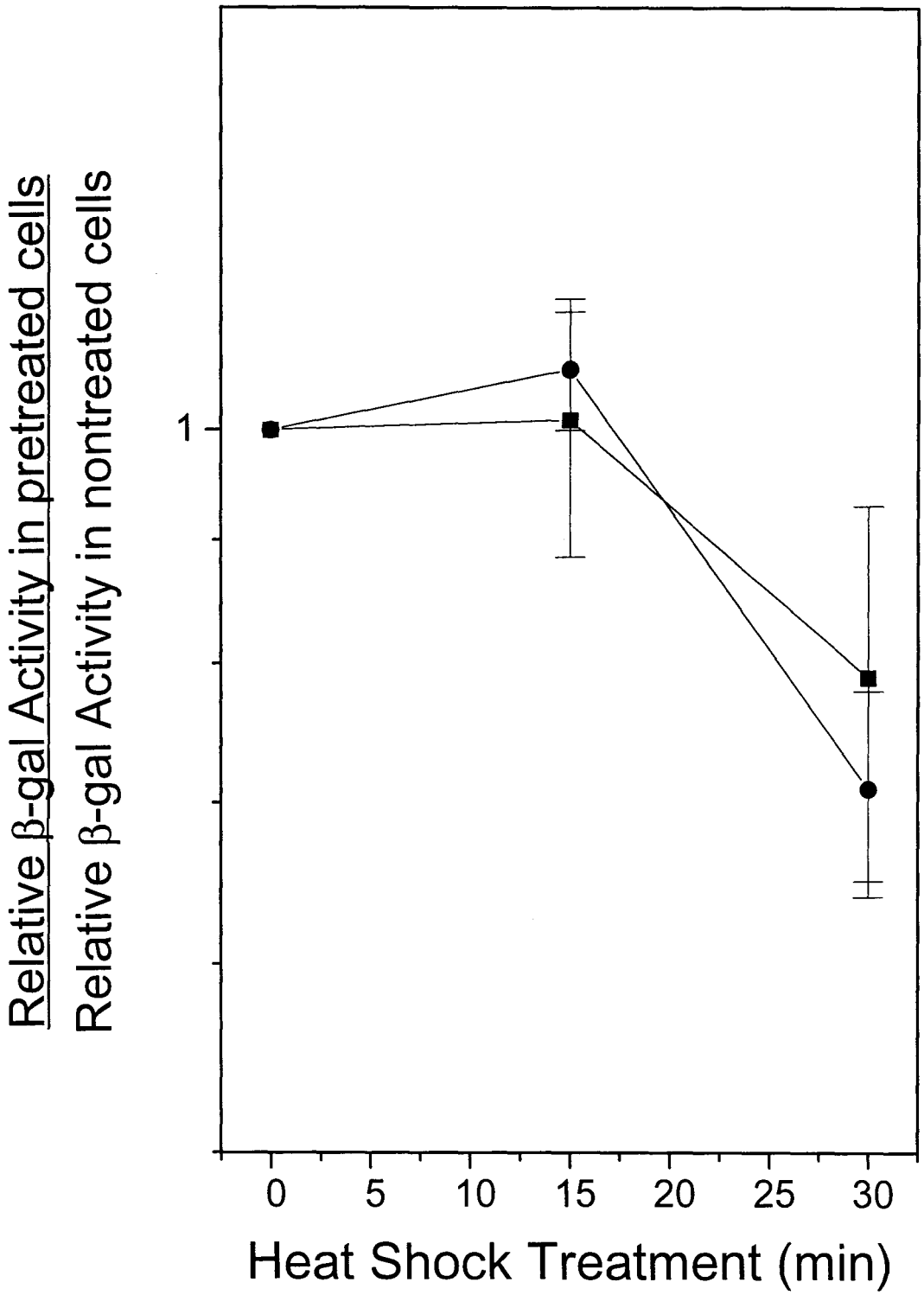
Relative β-gal Activity in pretreated cells
Relative β-gal Activity in nontreated cells
1
0
5
10
15
20
25
30
Heat Shock Treatment (min)

Figure 16: Enhanced expression of β-gal activity in Heat Shock treated RIF cells

Heat shock pretreated and untreated RIF-1 (-■-) and RIF-8A (-●-) cells were infected with non-irradiated AdCA35*lacZ*. Shows pooled results from two experiments each conducted in triplicate. β-Gal activity was measured at OD_{405}. Relative β-gal activity for undamaged reporter gene was calculated from the surviving fraction of reporter gene activity (treated cells/non-treated cells). Each data point represents the arithmetic mean ± standard error.

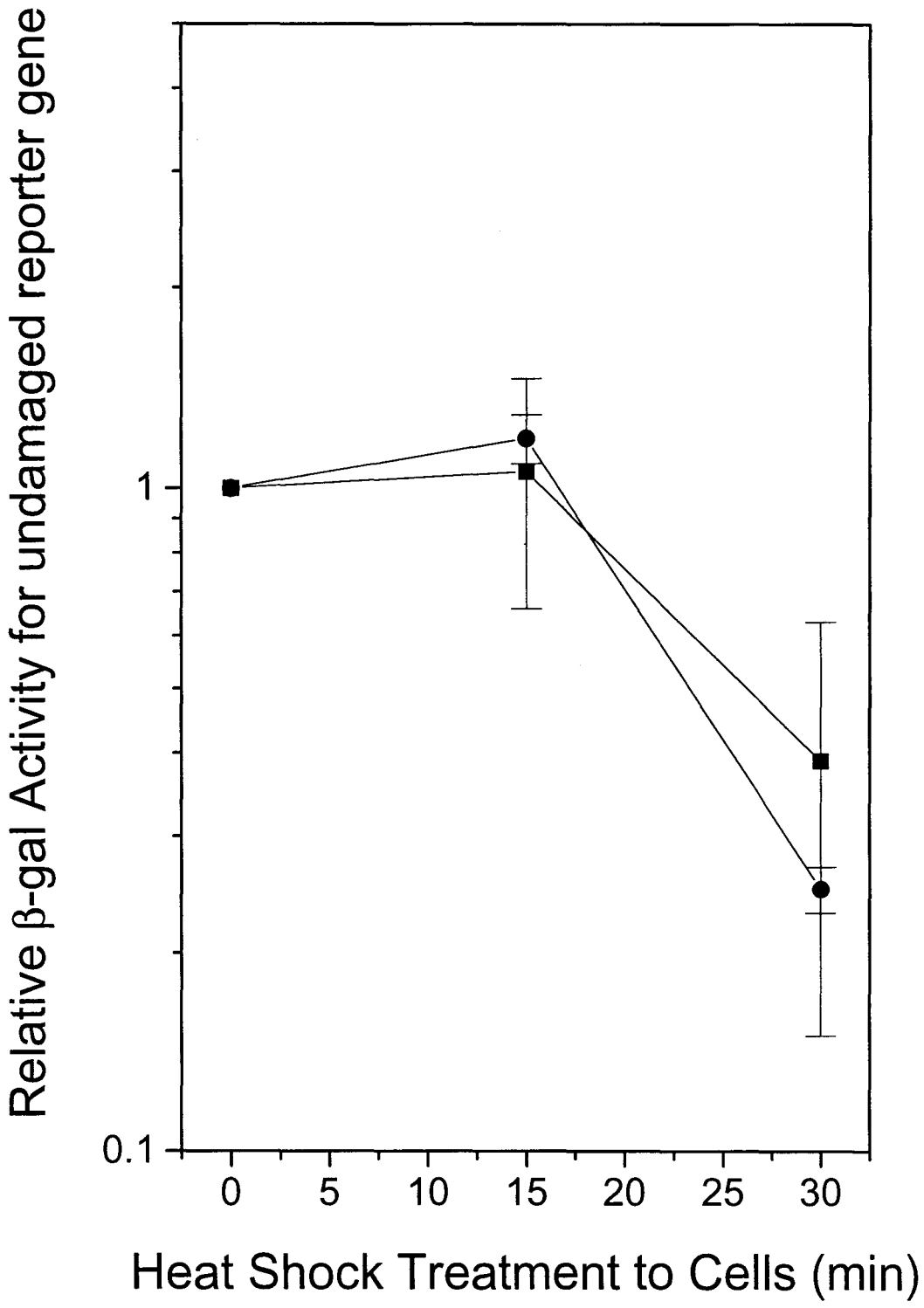
Relative β-gal Activity for undamaged reporter gene
1
0.1
0
5
10
15
20
25
30
Heat Shock Treatment to Cells (min)

TABLE 1: Relative survival of RIF cells and UV damaged AdCA35*lacZ* in RIF Cells

Treatment	Clonogenic Survival		ER at 300 J/m2 to virus		Enhanced Transcription	
	RIF -1	RIF-8A	RIF-1	RIF-8A	RIF-1	RIF-8A
Photofrin-PDT (μg/mL)	(3)	(3)	(2)	(2)	(2)	(2)
0	1	1	1	1	1	1
5	0.74 ± 0.08	0.86 ± 0.07	1.31 ± .06*	0.79 ± .04°	1.27 ± .58	1.59 ± .27
10	0.58 ± 0.07	0.65 ± 0.07	3.92 ± 1.25	1.0 ± .23	0.38 ± .21°	2.84 ± 1.26
15	0.02 ± .003	0.40 ± 0.08	7.92 ± 3.76	1.11 ± .21	0.07 ± .03°	2.74 ± 1.61
20	.002 ± .003	0.44 ± 0.20	5.47 ± .15*	0.37 ± .06°	0.04 ± .03°	2.27 ± 1.09
JM2929-PDT (μg/mL)	(3)	(3)	(2)	(2)	(2)	(2)
0	1	1	1	1	1	1
5	0.63 ± .03	0.63 ± .07	1.51 ± .18	1.04 ± 0.58	0.51 ± .09°	1.28 ± .86°
10	0.17 ± .05	0.19 ± .07	2.63 ± 1.68	0.78 ± 0.31	0.31 ± .04°	0.17 ± .06°
15	0.11 ± .02	0.08 ± .02	4.10 ± .59*	0.85 ± 0.70	0.16 ± .05°	0.11 ± .07°
20	0.01 ± .003	0.01 ± .004	1.80 ± 0.91	1.59 ± 0.10	0.16 ± .08°	0.13 ± .06°
UV (J/m^2)	(3)	(3)	(3)	(3)	(3)	(3)
0	1	1	1	1	1	1
2	-	-	0.82 ± 0.23	0.91 ± 0.09	1.55 ± 0.30	1.12 ± .15
5	0.81 ± 0.1	0.83 ± 0.09	1.57 ± .24*	1.20± .07*	1.06 ± 0.16	1.13 ± .24
10	0.29 ± 0.19	0.31 ± 0.14	0.93 ± 0.34	1.03 ± 0.08	2.16 ± 0.79	1.33 ± .17
20	0.03 ± 0.01	0.06 ± 0.02	0.74 ± 0.22	0.79 ± 0.09	2.11 ± 0.67	1.62 ± .06*
Cisplatin (μM)	(3)·	(3)·	(3)	(3)	(3)	(3)
0	1	1	1	1	1	1
12.5	≈0.007	≈0.11	0.97 ± 0.15	0.96 ± 0.18	1.11 ± 0.11	1.08 ± 0.02
Heat Shock at 43.5°C (min)	(3)	(3)	(3)	(3)	(3)	(3)
0	1	1	1	1	1	1
15	0.83 ± 0.11	0.92 ± 0.15	1.01 ± 0.12	1.06 ± 0.06	1.06 ± 0.4	1.19 ± 0.1
30	0.70 ± 0.04	0.60 ± 0.07	0.79 ± .14°	0.71 ± .07°	0.39 ± 0.4	0.25 ± .02°

( ) Number within brackets indicates the number of experiments conducted for each assay
·Data obtained from Moorehead et al. (1994)
* significantly greater than 1 ($p<0.05$) by 1 tailed test
° significantly less than 1 ($p<0.05$) by 1 tailed test

Discussion

Previous results suggest a cross-resistance to UV by Ph-PDT resistant RIF-8A cells shown by viral capacity (Di Prospero et al. 1996) and clonogenic survival (Roy et al. 1996). The viral capacity assay measures the ability of cells, which have been treated with a chemical or physical agent, to support viral growth (Di Prospero et al. 1996). The colony forming assay can measure a cell's ability to retain its reproductive integrity and thereby proliferate, following treatment with a chemical or physical agent (Hall 1994). HCR of virus is a sensitive and quantitative measure of the repair capacity of the host cell, not treated with a damaging agent, to repair and hence replicate damaged viral DNA induced by a number of physical and chemical agents (Parsons et al. 1986, Day et al. 1975, Rainbow 1989). In this work, the HCR of a UV damaged reporter gene was examined in RIF-1 and RIF-8A cells. Results show increased HCR of UV damaged AdCA35lacZ in RIF-1 cells compared to RIF-8A cells. This suggests increased repair of UV damage in an actively transcribed gene in RIF-1 cells compared to RIF-8A cells. This result also suggests that the capability for increased repair of UV damage in an actively transcribed gene does not correlate with increased survival of RIF-1 cells following UV exposure.

ER of virus involves the combination of HCR of damaged viral DNA, with cells that are pretreated with damaging agent (Parsons et al. 1986). ER in mammalian cells has been compared to the SOS response observed in bacteria (Dion and Hamelin 1987, Hilgers et al. 1989, Coppey and Menezes 1981). ER of a UV damaged DNA virus in

mammalian cells is suggested to result from an inducible repair pathway (Rainbow 1981). In our laboratory, ER by UV or heat shock (Francis and Rainbow 1995, McKay and Rainbow 1996) has been demonstrated in normal human fibroblasts as well as normal lung epithelial cells. In this work, we observed Ph-PDT enhanced reactivation of a UV damaged reporter gene in murine fibrosarcoma RIF-1 cells, but not in Ph-PDT resistant RIF-8A cells. This difference in enhanced reactivation was observed at a Ph-PDT dose that results in approximately 2% survival of RIF-1 cells, and 40% survival of RIF-8A cells. However, at this Ph-PDT dose, the expression of β-gal activity from non-irradiated reporter gene was significantly greater in Ph-PDT treated RIF-8A cells compared to RIF-1 cells. These results suggest that RIF-1 cells are more sensitive to Ph-PDT induced inhibition of transcription compared to RIF-8A cells. Inhibition of transcription in RIF-1 cells following Ph-PDT may be related to the enhanced reactivation of a UV damaged reporter gene in RIF-1 cells. Several other studies suggest a coupling of transcription termination to repair (Yamaizumi and Sugano 1994). It is possible that differences in cell cycle arrest following Ph-PDT may exist for RIF-1 and RIF-8A cells, and that such differences could result in the increased Ph-PDT resistance observed for RIF-8A cells compared to RIF-1 cells. Whereas, the presence of inducible repair and increased cell cycle block may be related to reduced survival for RIF-1 cells following Ph-PDT. It is also possible that following Ph-PDT, RIF-8A cells are more efficient in post-replication repair as compared to RIF-1 cells. Post-replication repair is a repair process whereby a cell's repair machinery bypasses DNA template damage, and the damaged DNA is still replicated. (Weaver and Hedrick 1989, Friedberg et al. 1995).

Previous results suggest no difference in survival between JM2929 -PDT treated RIF-1 and RIF-8A cells (Roy et al. 1996) However, at a JM2929-PDT dose that results in approximately 11% survival for RIF-1 cells and 8% survival for RIF-8A cells, RIF-1 cells showed an enhanced reactivation of a UV damaged reporter gene compared to RIF-8A cells. Yet, no difference in expression of β-gal activity from a non-damaged reporter gene was observed between RIF-1 and RIF-8A cells following a similar JM2929-PDT pretreatment dose to cells. Similar expressions of β-gal activity from a non-damaged reporter gene in JM2929-PDT treated RIF-1 and RIF-8A cells may be related to similar survival of RIF-1 and RIF-8A cells observed following JM2929-PDT treatment.

Ph-PDT resistant RIF-8A cells have shown a cross resistance to UV (Di Prospero et al. 1996, Roy et al. 1996). UV exposure of $5J/m^2$ results in approximately 81% survival of RIF-1 cells and 83% survival of RIF-8A cells. At a higher UV exposure, ($40J/m^2$) there is an approximate 10 fold difference in survival between RIF-8A and RIF-1 cells (results not shown). Results show increased reactivation of a UV damaged reporter gene (at UV exposure of $5J/m^2$) in RIF-1 cells compared to RIF-8a cells. Enhanced expression of β-gal activity from a non-damaged reporter gene was seen for both RIF-1 and RIF-8A cells following UV pretreatment. Similar to Ph-PDT results correlating presence of inducible repair and decreased survival in Ph-PDT treated RIF-1 cells, increased inducible repair in RIF-1 cells compared to RIF-8A cells following low UV exposure pretreatment may be related to the decreased survival of RIF-1 cells observed following UV treatment. This result also supports the suggestion of some

overlap in the type(s) of cellular damage induced by UV and Ph-PDT and/or an overlap in the pathways for the repair of UV and Ph-PDT damage in RIF cells (Di Prospero et al. 1996).

Previous work has shown a cross resistance of Ph-PDT resistant RIF-8A cells to cisplatin (Moorehead et al. 1994). Some researchers have examined host cell reactivation of cisplatin treated virus in different mammalian cells (Maynard et al. 1989, Zeng Rong et al. 1995, Jennerwein et al. 1991). However to our knowledge, there have not been any reports of enhanced reactivation studies in mammalian cells following cisplatin pretreatment. At a cisplatin dose that results in approximately 0.7% survival in RIF-1 cells and 11% survival in RIF-8A cells, no enhanced reactivation of a UV damaged reporter gene was seen for either RIF-1 or RIF-8A cells. Results show no enhanced expression of β-gal activity from non-damaged virus in RIF-1 or RIF-8A cells following pretreatment with a similar dose of cisplatin. It is possible that following cisplatin damage, morphological and functional changes in the mitochondria, rather than differences in the DNA repair, play a significant role in the cellular survival for RIF cells.

Over the range of hyperthermia doses examined, no difference in survival was observed for RIF-1 and RIF-8A cells. At a hyperthermia treatment dose that results in approximately 70% survival in RIF-1 cells and 60% survival in RIF-8A cells, decreased reactivation of a UV damaged reporter gene was seen for both RIF-1 and RIF-8A cells. Decreased expression of β-gal activity from a non-damaged reporter gene following a similar hyperthermia dose was observed for both RIF-1 and RIF-8A cells. Ph-PDT and

hyperthermia have been shown to cause similar types of damage to RIF-1 tumor cells, however the mechanism(s) leading to tumor cell damage are thought to be separate for these two modalities (Henderson et al. 1985). Absence of cross resistance of Ph-PDT resistant RIF-8A cells to hyperthermia supports the suggestion of separate mechanism(s) of damage induced by Ph-PDT and hyperthermia. Decreased reactivation of a UV damaged reporter gene and decreased expression of β-gal activity from a non-damaged reporter gene were observed for both RIF-1 and RIF-8A cells following hyperthermia pretreatment. However, it is possible that at higher doses of hyperthermia treatment, results for enhanced reactivation of a UV damaged virus and for enhanced expression of β-gal activity from a non-damaged reporter gene may differ between RIF-1 and RIF-8A cells. Further examination of heat shock ER for RIF-1 and RIF-8A cells following equitoxic treatments of Ph-PDT and hyperthermia is required.

These results suggest that the relation between survival, enhanced reactivation of a UV damaged reporter gene, and enhanced expression of β-gal activity from a non-damaged reporter gene varies in RIF-1 and RIF-8A cells depending on the damaging agent. However it is suggested that the decreased reactivation of a UV-damaged reporter gene in RIF-8A cells, compared to RIF-1 cells, may be related to the increased survival of RIF-8A cells following Ph-PDT and UV treatments.

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CHAPTER 5

SUMMARY

SUMMARY

In this work, we have examined the sensitivity of murine fibrosarcoma cells, with varying Ph-PDT sensitivities, to several different damaging agents. Results from the Hoechst Fluorochrome assay as well as the colony forming assay indicated no difference in survival between RIF-1 and RIF-8A cells following PDT mediated by the novel phthalocyanine sensitizer JM2929 (Table I). These results suggest that the cellular targets for JM2929-PDT and Ph-PDT are not identical in RIF-1 and RIF-8A cells. The Hoechst Fluorochrome assay results showed a cross resistance to JM2929-PDT for the Ph-PDT resistant CPR-C1 and RIF-P16CL8 cells (Table I). These results suggest that the cellular changes required for RIF-8A cells compared to CPR-C1 cells and RIF-P16CL8 cells to become resistant to Ph-PDT are different, or that additional changes have occurred in one or all of the Ph-PDT resistant variants, that affect their JM2929-PDT sensitivity.

Colony forming assay results of UV irradiated RIF-1 and RIF-8A cells obtained in this work support previous viral capacity assay results that indicated a cross-resistance to UV irradiation of Ph-PDT resistant RIF-8A cells (Table I). These results suggest that there may be similarities in the components of damage and/or similarities in the repair pathways of Ph-PDT and UV treatments in RIF-1 and RIF-8A cells.

In this work, colony forming assay results indicated a cross resistance of Ph-PDT resistant CPR-C1 cells, but not Ph-PDT resistant RIF-P16CL8 cells, to gamma radiation

(Table I). Ph-PDT resistant RIF-8A cells showed a significantly increased sensitivity to gamma-rays compared to RIF-1 parent cells (Table I). These results suggest that the cellular changes required for the RIF-8A cells to develop resistance to Ph-PDT interfere with the ability of these cells to recover from the cellular damage induced by gamma radiation. These results also suggest that the cellular changes required for RIF-8A cells compared to RIF-P16CL8 and CPR-C1 cells to become resistant to Ph-PDT are different, or that additional changes have occurred in one or all of the Ph-PDT resistant variants that affect their gamma-ray sensitivity. These results, as well as results obtained for the JM2929-PDT sensitivities of the Ph-PDT resistant variants, suggest that more than one mechanism of induced resistance to Ph-PDT exists in RIF cells.

Colony forming assay results of RIF-1 and RIF-8A cells treated with gamma-rays in the presence of Photofrin indicated that Photofrin increases gamma-ray sensitivity in RIF-1 cells, but not RIF-8A cells (Table II). Gamma-irradiation in the presence of JM2929 had no radiosensitizing effect on the survival of either RIF-1 or RIF-8A cells (Table II). This result suggests that Photofrin may act as a radiosensitizer in the clinical treatment of radioresistant tumors. This result also suggests that gamma-radiation and red light exposure in the presence of Photofrin both induce a type of damage which results in a greater lethality for RIF-1 compared to RIF-8A cells. It is possible that this type of damage can be repaired in RIF-8A, but not RIF-1 cells.

Colony forming results of RIF-1 and RIF-8A cells treated with Ph-PDT followed by hyperthermia indicated synergistic effects on the survival of RIF-8A cells, but not RIF-1 cells (Table III). No effect on the survival of RIF-1 and RIF-8A cells was observed

when cells were treated with JM2929-PDT followed by hyperthermia, compared to the survival of RIF-1 and RIF-8A cells treated with JM2929-PDT alone (Table I). These results suggest that in the clinical setting, combinational treatments using Ph-PDT and hyperthermia may be of added benefit in the treatment of Ph-PDT resistant tumors.

HCR of a UV damaged reporter gene was used to determine the differences in DNA repair between RIF-1 and RIF-8A cells. Results indicated that the HCR of a UV-damaged reporter gene was increased in RIF-1 compared to RIF-8A cells (Table IV). These results suggest that the increased survival of RIF-8A cells compared to RIF-1 cells following UV exposure does not result from an increased capability for repair of UV damage in an actively transcribed gene.

Results indicated enhanced reactivation of a UV damaged reporter gene in Ph-PDT pretreated RIF-1 cells, but not RIF-8A cells. However, enhanced expression of β-gal activity from a non-damaged reporter gene was observed in Ph-PDT pretreated RIF-8A cells, but not RIF-1 cells (Tables IV & V). These results suggest that the decreased capability for DNA repair of an actively transcribed gene, and the transcriptional enhancement of an actively transcribed gene in Ph-PDT pretreated RIF-8A cells, may be related to the resistance observed by RIF-8A cells treated with Ph-PDT.

A decreased level of reactivation of a UV damaged reporter gene was observed in Ph-PDT as well as UV pretreated RIF-8A cells compared to RIF-1 cells (Table IV). These results suggest that the decreased level of inducible repair of an actively transcribed gene may be related to Ph-PDT and UV resistance observed in RIF-8A cells compared to RIF-1 cells.

ER of a UV damaged reporter gene, as well as the expression of β-gal activity from a non-damaged reporter gene, in RIF-1 and RIF-8A cells pretreated with JM2929-PDT, cisplatin, or hyperthermia were examined (Table IV & V). Results suggest that the relation between survival, DNA repair and transcriptional enhancement of an actively transcribed gene varies in RIF-1 and RIF-8A cells, depending on the damaging agent employed.

Table I: Sensitivity of RIF cells to Different Damaging Agents

Damaging Agent	RIF-1	RIF-8A	RIF-1G	RIF-P16CL8	CPR-C1
Ph-PDT (HFA)	+	+++	ND	ND	ND
Ph-PDT (CFA)	+	+++	+°	+++°	++++*
JM-PDT (HFA)	+	+	+	+++	++++
JM-PDT (CFA)	+	+	ND	ND	ND
UV light (CFA)	+	++	ND	ND	ND
Gamma-rays (CFA)	+++	+	+	+	+++
Heat Shock (CFA)	++	++	++°	++°	ND

Table II: Combined Photosensitizer and Gamma-ray Treatments in RIF cells

Combined PDT and Gamma-Rays	RIF-1	RIF-8A	RIF-1G	RIF-P16CL8	CPR-C1
Ph + Gamma rays (CFA)	+	+	ND	ND	ND
JM + Gamma rays (CFA)	+++	+	ND	ND	ND

Table III. Combined PDT and Heat Shock Treatments in RIF cells

Combined PDT and Heat Shock	RIF-1	RIF-8A	RIF-1G	RIF-P16CL8	CPR-C1
Heat Shock (CFA)	++	++	++°	++°	ND
Ph-PDT + Heat Shock (CFA)	++	+	ND	ND	ND
JM-PDT + Heat Shock (CFA)	++	++	ND	ND	ND

(HFA) Hoechst Fluorochrome Assay, (CFA) Colony Forming Assay, (Ph) Photofrin, (JM) JM2929,
(ND) not done, (+ ++ +++ ++++) relative sensitivity in increasing order of resistance
*(Luna personal communication)
°(Luna and Gomer 1991)

Table IV· HCR and ER of a UV damaged reporter gene in RIF cells

HCR and ER	RIF-1	RIF-8A
HCR	++	+
Ph-PDT ER	+++	
JM-PDT ER	++	
UV ER	++	+
Cisplatin ER		
Heat Shock ER	--	--

(HCR) Host Cell Reactivation, (ER) Enhanced Reactivation
(+ ++ +++) enhanced repair in order of increasing levels
( ) no enhanced repair, (--) decreased repair

Table V Enhanced Expression of a reporter gene in RIF cells

Enhanced Expression	RIF-1	RIF-8A
Ph-PDT EE		++
JM-PDT EE		
UV EE	+	+
Cisplatin EE		
Heat Shock EE	--	--

(EE) Enhanced expression
(+ ++) enhanced expression in order of increasing levels
(-) no enhanced expression, (--) decreased expression

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