AN EXAMINATION OF SENSITIVITY OF PHOTODYNAMIC THERAPY-RESISTANT HT29 CELLS TO ULTRAVIOLET RADIATION AND CISPLATIN

AN EXAMINATION OF SENSITIVITY OF PHOTODYNAMIC THERAPY-RESISTANT HT29 CELLS TO ULTRAVIOLET RADIATION AND CISPLATIN

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ABSTRACT

Photodynamic therapy (PDT) is a form of cancer treatment involving light, a photosensitizer and oxygen, whereby the photosensitizer is preferentially taken up by tumour cells, excited when exposed to light of the appropriate wavelength, and generates cytotoxic excited singlet oxygen that damages and destroys cells. Photofrin is the only approved photosensitizer for clinical use in treating esophageal and early and late lung cancers in the U.S., Canada and several other countries. Despite its effectiveness in treating some tumour types, Photofrin use has some limitations and thus photosensitizers are continuously being studied to find more efficient ways of killing tumour cells.

Previous reports have described the isolation of photodynamic therapy resistant human colon carcinoma HT29 cells. HT29/P14, HT29/A11 and HT29/N8 were isolated by repeated *in vitro* PDT treatment to the 1–10% survival level followed by regrowth of single surviving colonies using the photosensitizers Photofrin, Aluminium Phthalocyanine Tetrasulphonate (AlPcS4) and Nile Blue A respectively. These PDT resistant HT29 variants all display increased levels of BNip3, Bcl-2 and the heat shock protein 27 (Hsp 27), but decreased levels of Bax and the mutant HT29 p53 protein. Since mutant p53 and increased expression of Hsp27 and Bcl-2 and have been associated with resistance to various chemotherapeutic agents in some tumour cells, whereas Bax and BNip3 are potent inducers of apoptosis, it was considered of interest to examine the sensitivity of these PDT resistant HT29 variants to other cytotoxic agents. Cell sensitivity to ultraviolet (UV) A radiation (UVA), a mixture of UVA and UVB (UVA/B), UVC, or

cisplatin was determined by a comparison of the D_{37} values for clonogenic survival in the variants compared to that in parental HT29 cells.

The HT29 PDT resistant variants were not cross-resistant to cisplatin or UVC. In contrast, HT29/P14, HT29/A11 and HT29/N8 all showed a significant increase in cisplatin sensitivity, while HT29/A11 cells also showed a significant increase in UVC sensitivity. HT29/N8, and HT29/P14 both showed a significant increase in UVA resistance compared to HT29 cells whereas HT29/A11 did not. HT29/P14 was the only PDT-resistant cell line significantly cross-resistant to UVA/B relative to HT29. While HT29/P14 and HT29/A11 both showed a slight increase in resistance to Photofrin-mediated PDT compared to HT29/Parental, this increase was only significant for HT29/A11. However, HT29/N8 was significantly more sensitive to Photofrin-mediated PDT than HT29/Parental. To complicate matters, clonogenic variability was observed amongst the two HT29 sources examined, since one of the original HT29 cell lines showed a significantly higher resistance to Photofrin-mediated PDT compared to the other parental HT29 cells that were used to derive the PDT-resistant cell lines.

To examine if the differences in sensitivity of the PDT-resistant cell lines compared to parental HT29 cells in response to cisplatin and UV radiation were due to differences in DNA repair, host cell reactivation (HCR) experiments were performed with a UVC damaged β-galactosidase reporter gene from the adenovirus Ad5HCMVSp1*LacZ*. HCR of the UV-damaged reporter gene was reduced in HT29/A11 (the cell line most sensitive to UVC) compared to the parental HT29 cells at high multiplicities of infection of the virus. This suggests the possibility of a decreased DNA

repair capacity for HT29/A11 cells. However, due to differences in cellular morphology between HT29 and HT29/A11 cells, as well as possible differences in expression of the reporter gene, it was inconclusive that the difference in HCR reflects a true difference in DNA repair between HT29 and HT29/A11 cells.

Hsp27 over expression alone was not responsible for the increased cisplatin sensitivity of the HT29 PDT resistant variants since there was no correlation of Hsp27 protein expression levels to $1/D_{37}$ (used as a measure of sensitivity), for the cisplatin colony survival assays. In addition, Hsp27 protein expression levels did not correlate with UVC, cisplatin or UVA sensitivity suggesting that Hsp27 may be uniquely involved in making cells more resistant to PDT. p53 but not BNip3 protein levels correlated with sensitivity of cells to UVA, whereas no correlation was observed between p53 or Hsp27 protein expression levels and UVC sensitivity. p53 and p21 protein levels were not altered in either parental HT29 or the HT29/P14 PDT-resistant variant following UVC and cisplatin exposure, respectively. In addition, introduction of wild-type p53 (using infection of a replication deficient adenovirus vector encoding the wild-type p53 gene), into parental HT29 or the PDT-resistant HT29/P14 variant, had no effect on cisplatin sensitivity compared to cells infected with a control adenovirus vector expressing the LacZ gene. Taken together, these results suggest that the increased sensitivity of the PDT resistant variants to cisplatin did not result from differences in p53-dependent cisplatin-induced cell cycle arrest.

A strong correlation of cellular cisplatin sensitivity to the ratio of BNip3 to p53 protein levels, suggests that alterations in the expression of several different genes,

including a reduced expression of the mutant HT29 p53 protein and an increased expression of BNip3, contribute to the increased cisplatin sensitivity of the HT29 PDT resistant variants. It has been reported previously that apoptosis induced by BNip3 is significantly inhibited by both wild type and mutated p53. Since pro-apoptotic BNip3 is over expressed in all three PDT-resistant HT29 cell lines, and BNip3/p53 protein expression levels were correlated to cisplatin sensitivity, this suggests that cisplatin kills HT29 cells through a BNip3-mediated apoptotic pathway.

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I would like to thank my supervisor, Dr. A. J. Rainbow for giving me the opportunity to work in his lab, and for his support, help, guidance, encouragement and patience throughout this experience. I especially want to thank him for teaching me about science, and a little about life too. I would also like to thank Dr. G. Singh for sitting on my supervisory committee and for providing helpful feedback and new ideas, and Dr. J. Quinn for serving on my thesis defence. I would also like to thank Hong Wang for her help with various experiments. Thank you to Dr. T. Finan and Dr. E. Weretilnyk, for the much needed and appreciated support, and encouragement.

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LIST OF ABBREVIATIONS

Ad	adenovirus
$AlPcS_4$	aluminum phthalocyanine tetrasulphonate
BER	base excision repair
BH	Bcl-2 homology
BNip3	Bcl-2 and Nineteen kDa interacting protein-3
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CPD	cyclobutane pyrimidine dimer
Cys	cysteine
DNA	deoxyribonucleic acid
D_{37}	dose required to give a SF of 0.37 (e^{-1})
FBS	foetal bovine serum
FDA	U.S. Food and Drug Administration
GGR	global genome repair
Glu	glutamate
Gly	glycine
GSH	glutathione
HHRAD23B	human homologue of the S. serevisiae protein 23
HCMV	human cytomegalovirus
HPD	haematoporphyrin derivative
Hsp	heat shock protein
lacZ	β-galactosidase
MMR	mismatch repair
MOI	multiplicity of infection
NBA	Nile Blue A
NER	nucleotide excision repair
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDT	photodynamic therapy
PF	photofrin
PKC	protein kinase C
PP	photoproduct
RPA	replication protein A
SDS	sodium dodecyl sulphate
SF	surviving fraction
TFIIH	transcription factor II H
TCR	transcription couple repair
UV	ultra violet
UVER	UV enhanced reactivation

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INTRODUCTION

INTRODUCTION

1. Cancer and Treatment

Cancer is a disease resulting from uncontrolled cell growth. It is initiated when a cell accumulates errors in some regulatory pathway, which gives it a survival advantage over the rest of the population of cells, allowing it to multiply uncontrollably. Cancer kills by disrupting function of vital organs, and is a major cause of death from disease in the human population. The treatment of cancer involves removing or killing the tumour cells while trying to avoid damage to healthy cells. While surgery is often done to remove cancerous tumours, patients are usually also given additional (adjuvant) treatment in the form of chemotherapy or radiotherapy to kill any cells which may have metastasized or not been removed by surgery. Unfortunately, chemotherapy and radiation therapy can also have toxic side effects on healthy cells. Thus patients undergoing treatment often experience symptoms of sickness, hair loss, fatigue and become immuno-compromised. Because of the DNA damaging properties of adjuvant treatments, there is also a risk of inducing cancer in healthy cells. For these reasons, researchers are seeking alternative forms of cancer treatment that are more effective and less toxic to cancer patients. (For review see King, 1996).

2. Photodynamic Therapy

Photodynamic therapy (PDT) is a form of cancer treatment involving light, a photosensitizer and oxygen (For review see Moor 2000, Oleinick and Evans 1998 and Dougherty *et al.*, 1998). The photosensitizer is preferentially taken up by tumour cells, excited when exposed to light of the appropriate wavelength, and generates cytotoxic excited singlet oxygen that damages and destroys cells (Weishaupt *et al.*, 1976).

PDT was first discovered at the turn of the century by Raab, who found that paramecia treated with acridine dye were killed by light. Since then, in 1961, Lipson and colleagues found that haematoporphyrin derivative (HPD) could be used for fluorescent detection of tumour cells. Eventually, this lead to aid in tumour detection and diagnosis. In 1975, Dougherty first reported the eradication of transplanted animal tumours with HPD and red light while sparing the surrounding tissue (as reviewed by Hsi *et al.*, 1999).

In recent years, the U.S. Food and Drug Administration (FDA) has approved the use of PDT with a particular sensitizing agent called Photofrin (PF) to treat certain forms of cancer. It has been approved for clinical use in esophagus and early and late lung cancer in the U.S., Canada and several other countries. Further research is being done into using PDT to treat other cancers easily accessible by light sources, such as cancers of the bladder, gastrointestine, head and neck, eye, ovary, and skin (As reviewed by Hsi *et al.*, 1999; Moor, 2000; and Oleinick and Evans, 1998). PDT is also being researched as a treatment for various non-oncological diseases such as age-related macular degeneration, and atherosclerotic plaque formation, and as a means of sterilizing cellular blood products (as reviewed by Moor, 2000).

PDT exerts its effects on cancer tumours in three ways. Firstly, PDT can directly kill tumour cells through apoptosis or necrosis. Secondly, PDT can cause damage to the surrounding vasculature, including blood flow stasis, vascular collapse and/or vascular leakage, ultimately leading to the tumour having severe hypoxia. In addition, PDT can induce an immune reponse through the release of cytokines and other inflammatory mediators (as reviewed by Oleinick and Evans, 1998). It is the combination of all three components which can provide long-term tumour control (Dougherty *et al.*, 1998).

The mode of cell death by PDT depends on the experimental conditions, such as dose of PDT, and the intracellular localization of the photosensitizer. Insights into the mechanisms of tumour response and the mode of tumour cell death after PDT may lead to ways that will maximize the efficacy of PDT treatment.

2.1. Photosensitization

The application of light of the appropriate wavelength excites the photosensitizer which can undergo one of two reactions: type I and type II photo-oxidation (as reviewed by Foote, 1991; Hsi *et al.*, 1999; and Moor, 2000). Type I photo-oxidation is when the excited photosensitizer reacts directly with a biomolecule (for example a membrane lipid) either through the transfer of hydrogen or an electron, to form a free radical. The free radical further reacts with molecular oxygen to create oxygenated products such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) or superoxide radicals (O_2^-). These oxygenated products can undergo radical chain reactions ultimately resulting in cellular damage. Type II photo-oxidation results when the excited photosensitizer interacts with

molecular oxygen to form a singlet oxygen ($^1O_2$). Singlet oxygen is a non-radical but highly reactive oxidative species, which undergoes further reaction with substrates susceptable to oxidation. $^1O_2$ has a short lifespan of less than 0.04 microseconds in biological systems and therefore a short radius of action (<0.02μm) (Moan and Berg, 1991). This implies that the damage to the cell must occur in close proximity to the distribution of the sensitizer within the cell. The interaction of singlet oxygen or oxygen radicals with intracellular targets leads to damage in cell membranes, mitochondria, lysosomes and nuclei. When there is little oxygen present (oxygen levels are <2%), cells become PDT-resistant (Mitchell *et al.*, 1985; and Lee See *et al.*, 1984).

2.2. Photosensitizers

The ideal photosensitizer must have the following properties: selective uptake by the tumour, high quantum yields of singlet oxygen, sufficient tissue penetration, and photolability (as reviewed by Pass, 1993). The more selectively the photosensitizer is taken up by the tumour, the less phototoxicity the non-tumour cells will experience during and after the PDT treatment upon light exposure. Photolability is the ability of normal tissue to clear the photosensitizer. The rapid degradation of the photosensitizer in the body is essential so that patients do not experience prolonged photosensitivity.

Each photosensitizer has a unique light absorption spectrum, usually with a maximum absorption falling at a wavelength in the red light region of the visible light spectrum. Red light is not appreciably absorbed by the skin and is therefore the light colour of choice for PDT and determines which photosensitizers are suitable for PDT.

Photofrin absorbs most intensely in the blue wavelength of light, and has only a very weak absorption maximum at 630 nm, but longer wavelength light penetrates deeper into tissues. Second generation photosensitizers are therefore being designed to absorb light at longer wavelengths such as 650-850 nm. These allow tumours to be penetrated up to 30% deeper than photofrin-mediated PDT allows (as reviewed by Moore *et al.*, 1997; and Pass, 1993). Many of the second-generation photosensitizers are porphyrin-based or related and some examples include benzoporphyrin derivatives (BPD), tin-etiopurpurin (SnET2), lutetium texaphyrin (LuTex), and protoporphyrin IX (PPIX) (As reviewed by Oleinick and Evans, 1998; Dougherty *et al.*, 1998; Hsi *et al.*, 1999).

Photosensitizers show various sites of intracellular localization upon uptake and photosensitization. These include plasma membranes, nuclear membranes, lysosomes, Golgi apparatus, endoplasmic reticulum and probably most importantly the mitochondria. The site of localization can offer clues as to how that photosensitizer functions to destroy the cell. Also, identifying the targets of photosensitizers can help in drug development.

2.2.*i.* *Photofrin*

As mentioned previously, Photofrin is the only approved photosensitizer by the U.S. Food and Drug Administration (FDA). It has been in approved clinical use in esophagus and early and late lung cancer in the U.S., Canada and several other countries. Photofrin is a partially purified, complex mixture of porphyrin monomers and oligomers that has been derived from hematoporphyrin (Oleinick and Evans, 1998) (see Figure 1).

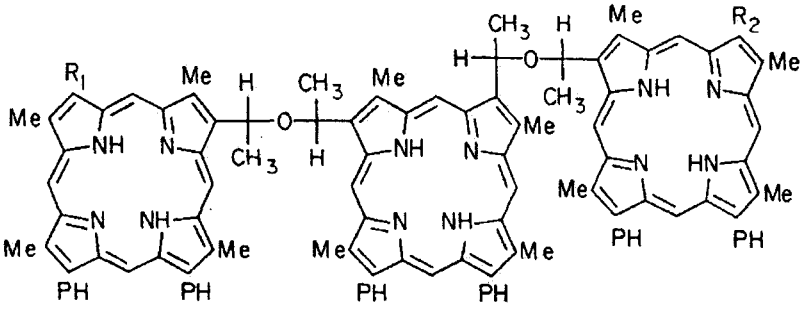


Figure 1: The major component of Photofrin, a porphyrin trimer. R1 and R2 can be $CH(OH)CH_3$ or $HC = CH_2$. PH represents $(CH_2)2COOH$. (From Dougherty and Marcus, 1992).

Photofrin first localizes to the plasma membrane and after several hours of incubation, to the nuclear membrane, lysosomes and specifically the mitochondria (Shulok *et al.*, 1990). Specifically, if radiation-induced fibrosarcoma (RIF) cells are incubated *in vitro* with Photofrin for 1 hour, Photofrin is found localized mostly in the plasma membrane. However, after 18 hours incubation in the presence of Photofrin, the drug is found localized mostly in the mitochondria (Wilson *et al.*, 1997).

The mitochondria have repeatedly been found to be the target of action of photofrin-mediated PDT (Hilf *et al.*, 1987; Salet and Moreno, 1990; and Morgan *et al.*, 2000). Several lines of evidence point to mitochondria as the primary targets of porphyrin-mediated PDT. Firstly, substitutions in the tetrapyrrole ring of porphyrin that increase mitochondrial localization, enhance its phototoxicity (Woodburn *et al.*, 1992). Secondly, Moreno and Salet demonstrated that after incubation with hematoporphyrin, micro-irradiation of subcellular areas rich in mitochondria resulted in cell death but cells survived irradiation of nuclei or haloplasms (Moreno and Salet, 1985). Porphyrins have

been shown to have a high affinity for the peripheral benzodiazepine receptor, which is a protein localized in the outer mitochondrial membrane (Ratcliffe and Matthews 1995; and Verma *et al.*, 1998). Porphyrin photosensitization results in detectable morphological changes in mitochondria such as swelling and complete disruption depending on the cell type examined. But eventually, other organelles such as the plasma membrane, nucleus and endoplasmic reticulum also end up with injury (as reviewed by Salet and Moreno, 1990).

Mitochondria house the enzymatic pathways which normally maintain energy synthesis for cellular growth, metabolism and repair (Richter and Kass, 1991). Porphyrin-meditiated PDT inactivates numerous mitochondrial enzymes (Hilf *et al.*, 1987), inhibits ATPase (Atlante *et al.*, 1990), and disrupts oxidative phosphorylation (Boegheim *et al.*, 1988). The impairment of ATP synthesis is probably one of the factors that lead to cell death (Gibson and Hilf, 1983).

To try and elucidate the importance of mitochondria as a cellular target of photorin-mediated PDT, several studies have been performed using pairs of cells with normal and deficient mitochondria (Morgan *et al.*, 2000; Sharkey *et al.*, 1993). Several groups have reported the isolation of RIF-1 cells resistant to Photofrin-mediated PDT through repeated Photofrin-mediated PDT exposure (Luna and Gomer, 1991; and Singh *et al.*, 1991). The PDT-resistant RIF-1 cells were named RIF-8A by Singh and colleagues (1991) and were later shown by the same group to have morphologically altered mitochondria that are smaller and more condensed than their RIF-1 counterparts (Sharkey et al., 1993). Photofrin was also seen localizing stronger in the inner mitochondrial

membranes of RIF-1 cells than RIF-8A cells (Wilson *et al.*, 1997). These studies suggest that Photofrin photosensitization occurs through the mitochondria and that resistance to PDT can be mediated through mitochondrial alterations.

Despite its effectiveness in treating some tumour types, Photofrin use has some limitations and thus photosensitizers are continuously being studied to find more efficient ways of killing tumour cells. Firstly, Photofrin use results in long lasting skin phototoxicity for up to 1-2 months after administration. Secondly, the complex chemical composition makes it more difficult to study. In addition, Photofrin has a relatively short wavelength absorption maximum of 630 nm (as reviewed by Oleinick and Evans, 1998; Dougherty *et al.*, 1998; Hsi *et al.*, 1999).

2.2.*ii.* *Aluminum Phthalocyanine Tetrasulphonate*

Aluminum phthalocyanine tetrasulphonate ($AlPcS_4$) is a second-generation photosensitizer that is part of a family of sulphonated phthalocyanines (see Figure 2A). The phthalocyanines can have a range of one to four sulphonated groups and the tetrasulphonated form is the most hydrophilic of the four forms (Vroenraets *et al.*, 2001). The chelation of phthalocyanine to a metal such as aluminum or zinc enhances its phototoxicity. $AlPcS_4$ has been said to be easier to produce and handle than Photofrin (Bown *et al.*, 1986). $AlPcS_4$ also has a more appropriate strong absorption peak than photofrin, falling in the red part of the spectrum at 675 nm (Barr *et al.*, 1987) (see Figure 2B). Also, cells sensitized with $AlPcS_4$ were shown to be much less sensitive to white light than cells sensitized with Photofrin, and $AlPcS_4$ is eliminated faster than Photofrin,

alleviating some of the clinical problems associated with prolonged photosensitivity after Photofrin sensitization (Bown *et al.*, 1986).

Upon photosensitization, $AlPcS_4$ has been shown to cause membrane damage and the plasma membrane is thought to be the primary site of action for this photosensitizer (as reviewed by Pass, 1993). However, the exact mechanism responsible for the cytotoxicity of $AlPcS_4$ is still unknown (Ben-Hur and Rosenthal, (1986). However, to confuse matters more, like other photosensitizers, several studies have shown the phthalocyanines can localize to other sites of the cell including lysosomes, Golgi apparatus and even the mitochondria depending on the incubation times and cell lines used (Fabris *et al.*, 2001; and Wood *et al.*, 1997).

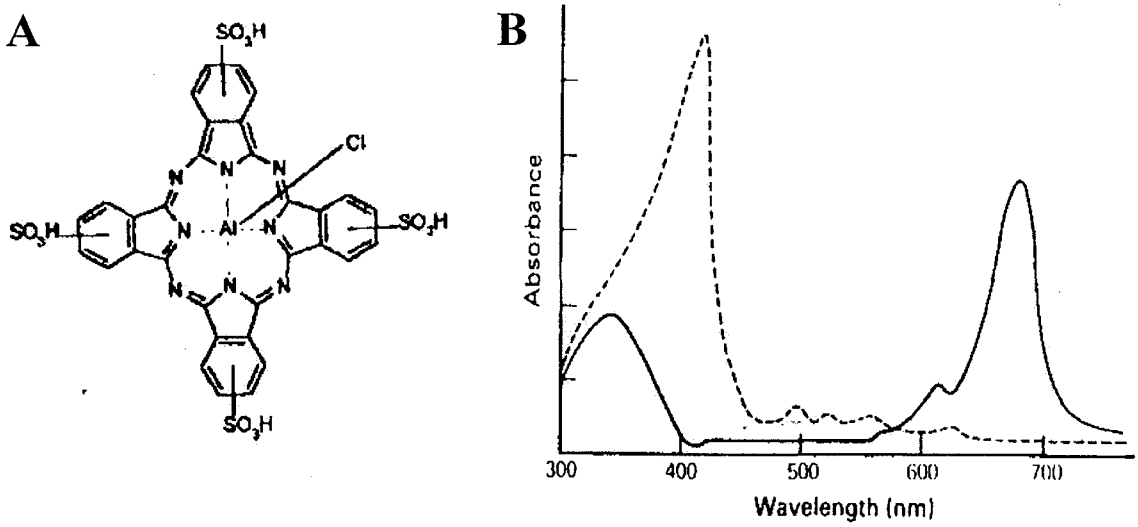


Figure 2: The structure of $AlPcS_4$ (A) (from Vrouenraets *et al.*, 2001) and the absorption spectra of a tri-sulphonated aluminimum phthalocyanine (solid line) and haematoporphyrin derivative (broken line) as measured at 5 μg/ml in FBS (B) (from Bown *et al.*, 1986).

2.2.*iii. Nile Blue A*

Nile Blue A (NBA) (see Figure 3) is a benzophenoxazine which is known to have high tumour selectivity and slows tumour growth in mice (Lewis *et al.*, 1949; and Riley *et al.*, 1948). Several studies suggest that Nile Blue A localizes to the lysosomes of cells and that the lysosomes may be the intracellular target for PDT killing of tumor cells (Lin *et al.*, 1991; 1993). The photodestruction of the lysosomes may release suicide enzymes and change the intracellular pH by releasing H+ ions and in combination, these may be responsible for causing the PDT induced cytotoxicity (Lin *et al.*, 1993). In addition to being phototoxic in human tumour cells, NBA also shows some dark toxicity in tumour cells and an extreme dark toxicity in normal human fibroblasts as well (Tong *et al.*, 2001). The later finding may limit its possible use in clinical treatment.

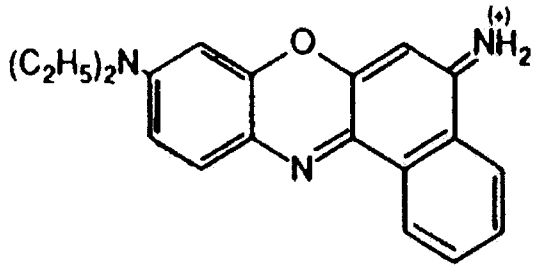


Figure 3: The structure of NBA (from Lin *et al.*, 1993).

2.3. Limitations of PDT

Although PDT may have milder side effects on the patient than other adjuvant therapies, the use of PDT is limited in that it can only be used on light accessible, localized tumours (not on metastases). Another limitation of *in vivo* PDT treatment is the

inhomogeneous distribution of the photosensitizer within the tumour (as reviewed by Dougherty *et al.*, 1998). Photosensitizer accumulation and tumour cell kill decrease proportionately with the distance from the vascular supply to the tumour (Korbelik and Krosl, 1994). A third limitation is seen with the limited oxygen available in tumours. Lack of oxygen severely limits the effectiveness of PDT treatment. Not only are some tumours already less oxygenated than normal tissue due to lack of vasculature, but PDT treatment also consumes any available oxygen as well as altering the microvasculature such that oxygen levels go down. One way to counteract this phenomenon is by lowering the fluence rate of the light used such that the oxygen consumption is slowed down, thus maintaining tumour oxygen levels during treatment (as reviewed by Pass, 1993). Also, second-generation photosensitizers are being designed that will exert smaller effects on the microvasculature (Dougherty *et al.*, 1998). Another reason why suboptimal PDT responses may occur is due to an inadequate light distribution and photosensitizer photobleaching (Ferrario *et al.*, 2000). Research is ongoing as to how to optimize the use of PDT for increased cancer cure-rates.

3. Cisplatin

Cisplatin is a platinum-containing compound that is a potent anticancer agent (for reviews see Demke *et al.*, 2000; Fuertes *et al.*, 2003; Gonzalez *et al.*, 2001; and Kartalou and Essigmann, 2001). Cisplatin is highly effective in the treatment of testicular and ovarian cancers and is also used to treat head and neck oesophageal, lung, and bladder cancer (Giaccone, 2000; and as reviewed by Fuertes *et al.*, 2003). However, some

tumours such as colorectal and nonsmall cell lung cancers show intrinsic resistance to cisplatin, and other tumours such as ovarian and small cell lung cancers can develop an acquired resistance after the initial cisplatin treatment. Also, cisplatin is toxic to non-tumour cells and because of its DNA damaging properties, can also induce cancer in healthy cells. As a result, cisplatin has been biochemically modified to try to overcome resistance and toxicity complications in tumour treatments. There are four biochemically altered forms of platinum drugs which include cisplatin, carboplatin, oxaloplatin, and nedaplatin (see Figure 4) currently in clinical use for the treatment of various tumour types (Judson and Kelland, 2000). Carboplatin is less toxic than cisplatin, and oxaloplatin is effective in treating cells that are resistant to cisplatin.

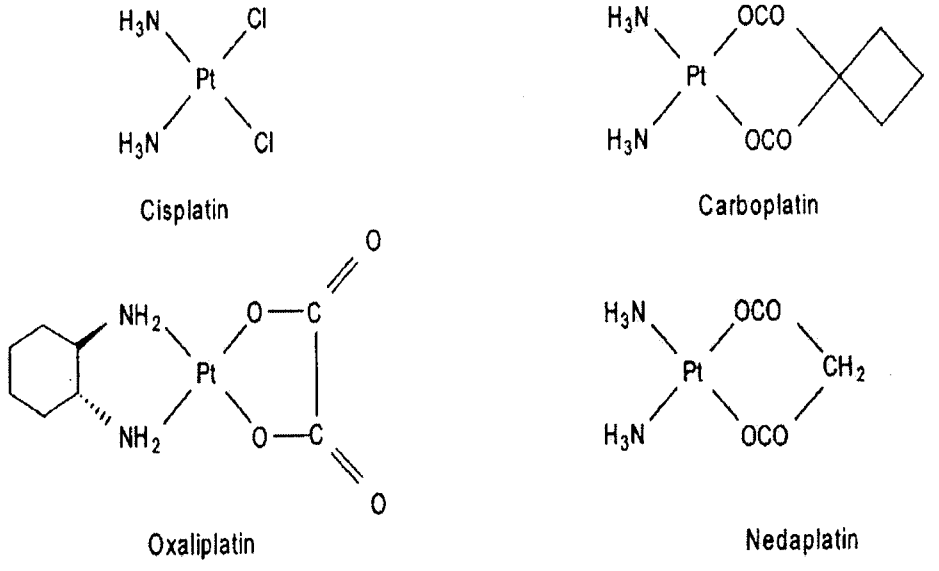


Figure 4: The structures of the four platinum drugs currently registered for clinical use: cisplatin, carbopolatin, oxaloplatin and nedaplatin (from Fuertes *et al.*, 2003).

Cisplatin acts by forming platinum adducts with DNA, RNA and protein. The ammonia groups are strongly coordinated to platinum, whereas the chloride ligands are easily substituted by water molecules. The extracellular environment has a very high chloride ion concentration (~100 mM) in which cisplatin remains coordinated to its two chloride ligands. When cisplatin enters the cell where the chloride ion concentration is low (~4 mM), its chloride ions are replace by water, generating a positively charged aquated species. This aquated form of cisplatin is very reactive towards nucleophile centers since H_20 makes a better leaving group than Cl^-, and forms DNA, RNA and protein adducts (see Figure 5). Cisplatin mostly forms intrastrand and some interstrand DNA crosslinks and monofunctional adducts. All of the cisplatin adducts bend and unwind DNA which ultimately causes the intereference of DNA replication and transcription (as reviewed by Kartalou and Essigmann, 2001). Cisplatin not only targets genomic DNA but also binds mitochondrial DNA.

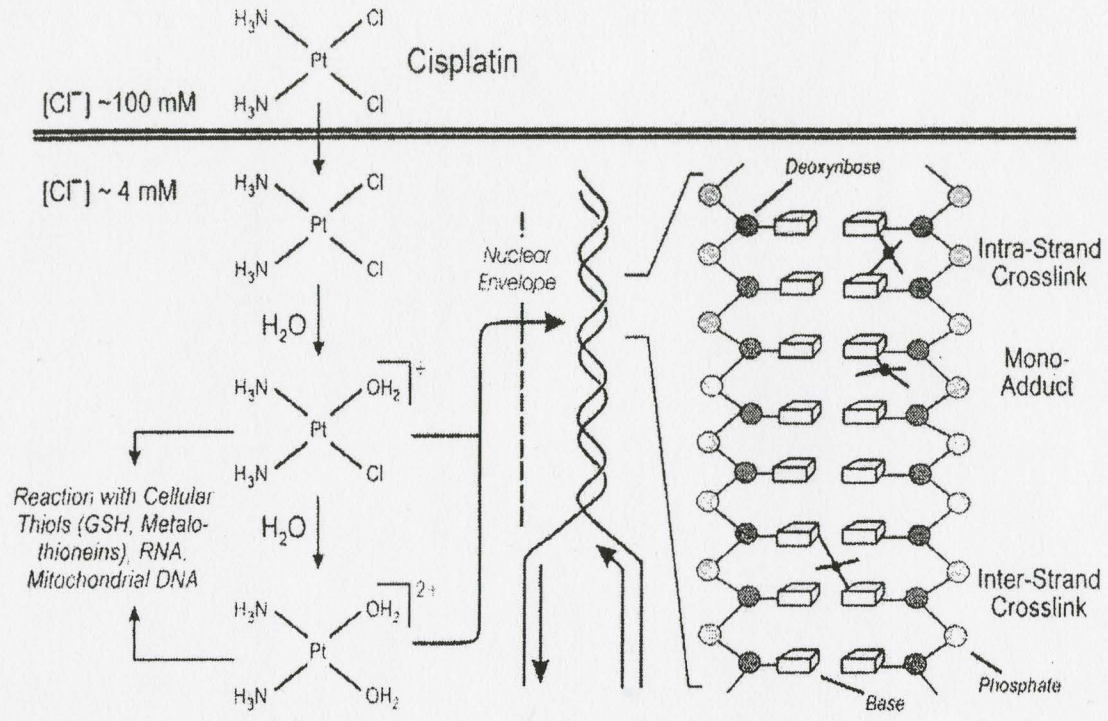


Figure 5: Cisplatin adduct formation inside cells (from Kartalou and Essigmann, 2001).

One possible mechanism by which cisplatin-DNA adducts kill cells is through the induction of programmed cell death also known as apoptosis (as reviewed by Gonzalez *et al.*, 2001). However, apoptosis is not the sole means of cell killing by cisplatin since cells killed by passive injury through necrosis and apoptotic cells have both been found in the same population of cells treated with cisplatin (Pestell *et al.*, 2000).

While cisplatin's effect on DNA has been studied quite extensively, the effect of cisplatin on cellular proteins is largely unknown. However, only 5-10% of covalently bound cell-associated cisplatin is found in the DNA fraction, while 75-85% is found

bound to proteins (Akaboshi *et al.*, 1992; and 1994). In addition, cisplatin may bind phospholipids and phosphatidylserine in the cell membrane, and cytoskeletal microfilaments, thiol containing proteins, and RNA in the cytoplasm (as reviewed by Fuertes *et al.*, 2003). Experimental evidence suggests that protein damage rather than DNA damage may be responsible for activating apoptotic pathways in response to cisplatin (Kruidering *et al.*, 1998).

There are multiple ways in which cells can become resistant to cisplatin (as reviewed by Dempke *et al.*, 2000; Gonzalez *et al.*, 2001; and Kartalou and Essigmann, 2001). They include reduced intracellular drug accumulation, increased repair of platinum-DNA adducts, inactivation of cisplatin by thiol species, and alterations in apoptotic proteins. Intracellular cisplatin accumulation can be reduced through decreased drug uptake or increased drug efflux. However, cisplatin is not a substrate for the P-glycoprotein (the *mdr* gene product) which is a drug efflux pump over expressed by many multi-drug resistant cell lines (as reviewed by Kartalou and Essigmann, 2001).

Intrastrand DNA platinum adducts are believed to be removed from cellular DNA by NER (Bulmer *et al.*, 1996). Evidence of this lies in the observation that cell lines deficient in NER proteins, such as XPA, are more sensitive to cisplatin than their corresponding wild type cell lines (Dijt *et al.*, 1988). In addition, XPA mRNA is expressed at higher levels in ovarian tumours from patients that are resistant to cisplatin therapy than in cisplatin responsive patients (Dabholkar *et al.*, 1994), and cisplatin-sensitive testicular tumour cells express very low levels of XPA and ERCC1-XPF (Koberle *et al.*, 1999). Altered ERCC1 levels have also been implicated in cisplatin

resistance (Bulmer *et al.*, 1996). Glutathione (GSH) is a thiol-containing species comprised of a tripeptide of glutamate (Glu), cysteine (Cys), and glycine (Gly). GSH reacts with cisplatin to form a deactivated conjugate that is excreted by pumps, thereby lowering intracellular cisplatin levels. Cisplatin resistance has often correlated with high intracellular GSH levels in tumours and GSH depletion has been shown to sensitize cells to cisplatin (as reviewed by Fuertes *et al.*, 2003). The mechanism by which cisplatin triggers apoptosis in cells still is not understood. However, a significant amount of cisplatin resistance cases are the result of a defective apoptotic program in cells (as reviewed by Gonzalez *et al.*, 2001).

4. Ultraviolet Radiation

Ultraviolet (UV) radiation from sunlight is a skin cancer causing agent which can be divided into 3 different wavelength regions (see Figure 6): short-wavelength UVC (200-280 nm), medium-wavelength UVB (280-320 nm) and long-wavelength UVA (320-400 nm). UVC radiation damages DNA in cells by causing cyclobutane pyrimidine dimer (CPD) formation and generating (6-4) photoproducts (PP) which are both repaired by nucleotide excision repair (Mitchell, 1988; and as reviewed by Hoeijmakers, 1993). Many investigators use UVC to study DNA damage and repair processes. However, due to the absorption by the atmosphere, UVC does not actually reach the earth. Therefore, the major relevant carcinogenic components of sunlight are UVA and UVB (as reviewed by Wang *et al.*, 1998). UVB, like UVC, directly excites DNA molecules and mostly causes pyrimidine dimers (Kim *et al.*, 2002). In contrast, UVA can not directly excite

DNA but rather indirectly damages DNA by a photosensitized reaction through the formation of reactive oxygen species (Kim *et al.*, 2002). However, a recent study shows that UVA can also produce thymine dimers, although at a much lower frequency than UVB and UVC do (Kuluncsics *et al.*, 1999). These reactive oxygen species mainly oxidize guanine bases producing for example 8-hydroxyguinine (8-oxoG) (Douki *et al.*, 1999), a non bulky oxidative DNA base modification which is primarily repaired through base excision repair (Kim *et al.*, 2002). Due to the depletion of the ozone layer, which filters out much of the solar UVB, there is concern that the amount of UVB reaching the earth's surface is on the rise along with the incidences of skin cancer.

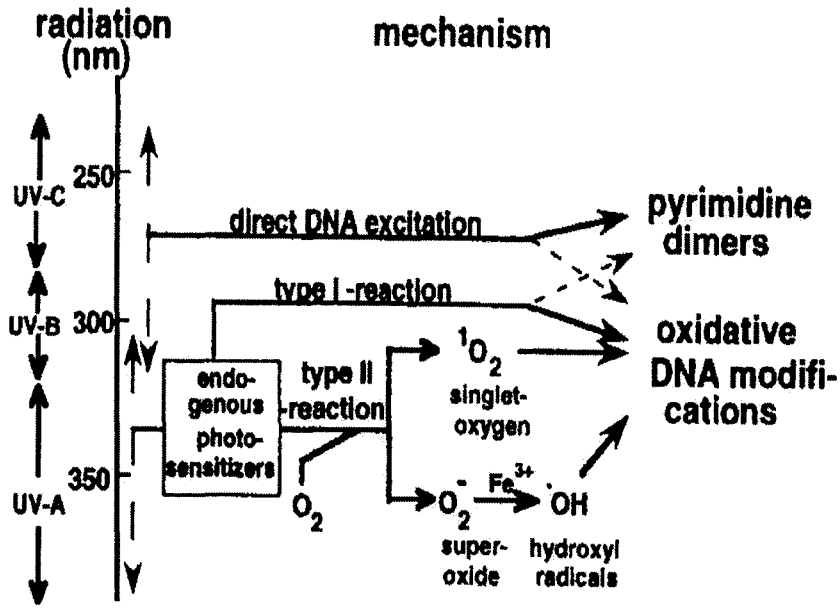


Figure 6: The direct and indirect mechanisms of pyrimidine dimer and oxidative damage formation in DNA by UV radiation (Kielbassa *et al.*, 1997).

5. DNA Damage and Repair

Numerous environmental components like UV radiation and chemotherapeutic agents such as cisplatin cause DNA damage. Damaged DNA can interfere with the process of DNA replication and transcription (As reviewed by Friedberg, 2001; and Hoeijmakers, 1993). More importantly, when left unrepaired, DNA damage leads to mutations in the genome which ultimately lead to cancer. A number of DNA repair systems have evolved in organisms that repair the lesions in cellular DNA continuously being formed through environmental insult. Nucleotide excision repair (NER) is the system responsible for removing bulky DNA lesions, while base excision repair (BER) removes damaged bases, and mismatch repair (MMR) removes incorrectly paired nucleotides (As reviewed by Friedberg, 2001).

5.1. Nucleotide Excision Repair

Nucleotide excision repair (NER) is the intracellular system responsible for removing bulky DNA lesions from the genome (For reviews see Friedberg, 2001; Hanawalt, 2002; Hoeijmakers, 1993; Sancar, 1994; and Wood, 1997). Examples of the lesions NER removes include cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4 PP) caused by UV light, benzo[a]pyrene-guanine products caused by smoking and the DNA intrastrand platinum adducts caused by the chemotherapeutic agent cisplatin. There are essentially five steps in NER: damage recognition, DNA incision a few bases away on either side of the lesion, exonuclease activity removing the

lesion-containing oligonucleotide, DNA synthesis by polymerase using the remaining undamaged strand as a template, and ligation to seal the nicks.

NER removes CPDs more efficiently from the actively transcribed genes than from the non-coding regions and can be subdivided into transcription-coupled repair (TCR) and global genome repair (GGR) (Bohr *et al.*, 1985). The component of NER that is coupled to RNA polymerase II is designated TCR and repairs the actively transcribed genes, while GGR repairs of the rest of the genome that is not actively being transcribed (Mellon *et al.*, 1987; Hanawalt, 1994).

A number of proteins are involved in each step of NER. Deficiencies in any of the NER proteins result in diseased individuals due to defects in DNA repair. There are three categories of human NER deficiency syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and Trichothiodystrophy (TTD). All NER deficiency syndrome patients are severely sensitive to sunlight and show neurodegeneration, but only XP patients also show a severe predisposition to cancer (as reviewed by Hoeijmakers, 1993; de Boer and Hoeijmakers, 2000; and Friedberg, 2001). XP is an autosomal recessive hereditary disease. In addition to being cancer prone, people with XP have dry skin (xeroderma) that is abnormally pigmented (pigmentosum). In addition to severe sun sensitivity and neurodegeneration, CS patients show developmental defects such as physical and mental retardation, retinal abnormalities, and also age prematurely.

Cell fusion studies have shown that different patients with an NER deficiency can complement each other's NER defects. Eight XP complementation groups have been identified through cell fusion studies designated XP-A through XP-G and XP-V

(Vermeulen *et al.*, 1997). Most XP groups have defects in both GGR and TCR. However, XP-C patients have a defect in GGR but a functional TCR (Venema *et al.*, 1991). The NER repair deficiencies seem to be most severe in XP groups A, B and G, and mildest in groups E and F (as reviewed by Hoeijmakers, 1993). XP-V stands for XP-variant and XP-V patients are thought to be deficient in post-replication repair and have mutations in the gene that codes for DNA polymerase η (Masutani *et al.*, 1999). There are only two CS complementation groups designated CS-A and CS-B. CS patients have a defect in NER that affects TCR, but their GGR remains functional (Venema *et al.*, 1990).

A model of both pathways of NER (GGR and TCR) is outlined in Figure 7. In GGR, the damaged DNA is recognized and partially opened by a heterodimer of XPC and the human homologue of the *S. serevisiae* protein 23 (HHRAD23B). In TCR, this is perfomed by CSB through the interaction with a stalled RNA polymerase. In both TCR and GGR, this is followed by the binding of another heterodimer of XPA and replication protein A (RPA), and the transcription factor II H (TFIIH). The DNA is locally unwound around the damage by TFIIH which is a subcomplex of the RNA polymerase II transcription initiation machinery composed of six subunits, and the helicases XPB and XPD. A bubble is generated in the DNA and ERCC1-XPF and XPG subsequently binds forming a complete NER multiprotein complex. XPG cleaves the DNA 3' of the damaged DNA while the ERCC1-XPF complex cleaves 5' of the damage site. The resulting 27-30 nucleotide long oligonucleotide is excised and replaced by DNA polymerase (δ or ε) and the remaining nicks are sealed by ligase). While TFIIH, XPG and the RPA/XPA/XPF/ERCC1 complex is used by both TCR and GGR, CSA and CSB are

only used in TCR, and the XPC/HHRAD23B complex is only used by GGR (for reviews see Wood, 1997; deBoer and Hoeijmakers, 2000; and Friedberg, 2001).

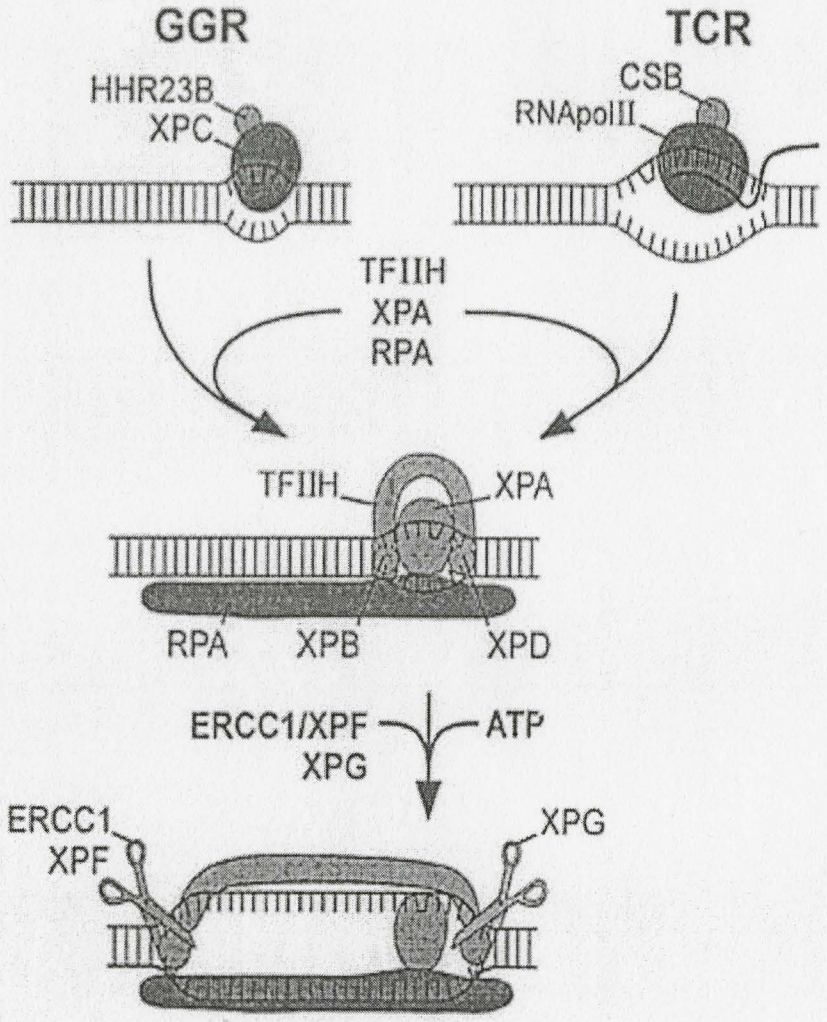


Figure 7: The steps involved in NER and the proteins responsible for each step (adapted from Winkler and Hoeijmakers, 1998).

5.2. Base Excision Repair

Base excision repair (BER) removes single damaged bases by means of multiple DNA-repair-specific enzymes called DNA glycosylases. There is a specific DNA glycosylase that recognizes and removes each type of damaged base. For example uracil DNA-glycosylase specifically recognizes uracil as an inappropriately incorporated base in DNA. Each glycosylase catalyzes hydrolysis of the N-glycosyl bond, excising the base from the DNA backbone leaving an apurinic or apirimidinic site (AP-site). AP endonuclease removes the AP-site and DNA polymerase and ligase fill in the DNA and seal the nick. (As reviewed by Friedberg, 2001).

5.3. Host Cell Reactivation (HCR)

There are several experimental techniques used to assess NER in mammalian cells. The earliest techniques involve treating cells with a DNA damaging agent and quantifying the number of DNA lesions, or measuring the amount of new DNA synthesis occurring as a result of DNA repair (termed unscheduled DNA synthesis). In these experiments however, the damage experienced by the cells during treatment with the DNA damaging agent, may not be isolated solely to the cellular DNA, making it difficult to isolate the mechanisms of constitutive DNA repair from inducible pathways.

A newer technique involves introducing a reporter gene containing damaged DNA into cells, and monitoring DNA repair through the protein expression levels of the reporter gene. The expression of the reporter gene in HCR is thought to reflect the repair of damaged DNA and a cell's HCR capacity is expressed as a ratio of the expression of

the damaged to undamaged reporter gene. Originally, this technique was performed using plasmids (Ganesan and Hanawalt, 1994; Smith *et al.*, 1995). However, the transfection procedure used to introduce plasmids into cells causes the activation of stress pathways similar to those induced by DNA damage (Renzing and Lane, 1995). In addition, primary human fibroblasts take up exogenous DNA far less efficiently than rodent or human tumour or transformed cells (Hoeijmakers, 1987; and Canaani *et al.*, 1986), such that most studies using HCR of damaged plasmids have used human tumour cells and transformed cells. Our lab and others have used adenovirus instead of plasmids to introduce the reporter gene into cells, allowing an examination of HCR in primary human fibroblasts (Francis and Rainbow, 2000; and Valerie and Singhal, 1995). The term 'host cell reactivation' is based on the analogy with the reactivation of damaged viruses by host cells (Ganesan and Hanawalt, 1999).

Human adenoviruses are increasingly being studied for use in gene transfer and therapy, for the development of viral vaccines, and for protein expression (Bett *et al.*, 1994). Adenovirus (Ad) is a non-enveloped virus containing double stranded DNA (Prescott *et al.*, 1996). Adenoviruses can be constructed such that a gene of interest is inserted into the Ad genome, usually replacing a deleted early region 1 (E1) or early region 3 (E3) (Bett et al., 1994). The E1 region is required for viral replication in most cell lines except 293 cells which express the necessary genes for viral replication (Graham *et al.*, 1977). Therefore E1 deletions render the adenovirus incapable of replicating in most cell types. In adenovirus used for HCR assays, the E1 region is replaced by a reporter gene such as the β-galactosidase gene under the human

cytomegalovirus (HCMV) promoter, and HCR is determined by measuring the expression level of this reporter gene following exposure to a DNA damaging agent (Francis and Rainbow, 2000). The HCMV promoter is used because it has been shown to direct high levels of gene expression in a wide variety of cell types (Xu *et al.*, 1995).

6. Apoptosis

Apoptosis is a mechanism whereby organisms initiate cell death by a process that is genetically encoded in their own genome (as reviewed by Gonzalez et al., 2001; Reed, 2000; Herr and Debatin, 2001). It differs from necrosis, a passive response to injury, in that apoptosis is an active process that requires ATP for its execution and often involves a gene called p53. Cell death by apoptosis is distinguished from necrosis by its characteristic unique morphological and biochemical features. These features include cell shrinkage, blebbing of the cell surface, loss of cell-cell contact, chromatin condensation and activation of endogenous endonucleases, dependence on ATP and on active protein synthesis, and lastly, the recognition by phagocytic cells (as reviewed by Gonzalez *et al.*, 2001). Apoptosis is necessary during fetal development and for tissue homeostasis. A cell's loss in the ability to undergo apoptosis can result in cancer.

Apoptosis can be divided into three phases: the initiation phase, effector phase, and the execution phase. The initiation phase occurs when a cell receives the stimulus, and any one of several possible pathways is engaged. The second effector phase is when all the initiating signals are integrated and a decision to live or die is made. The execution also known as the degradation phase, is an irreversible phase in which proteins

are autodigested and the DNA is cleaved (as reviewed by Fuertes *et al.*, 2003; and Gonzalez *et al.*, 2001). It is believed that the ratio of an anti-apoptotic Bcl-2 protein to the pro-apoptotic Bax protein finally determines whether or not a cell undergoes apoptosis (as discussed further below).

The execution phase of apoptosis is mediated by caspases (cysteine proteases cleaving at aspartic acid residues) and nucleases. Caspases cleave their substrates at aspartic acid residues contained within a tetrapeptide recognition motif. In apoptosis, initiator caspases (procaspase-8, -9, and –10) proteolytically activate downstream effector caspases (caspase-3, -6, and –7) which cleave specific substrates. The substrates of the effector caspases include protein kinases, signal transduction proteins, cytoskeleton and nuclear matrix proteins, chromatin-modifying and DNA repair proteins, and inhibitors of endonucleases and cleavage of these substrates gives cells their apoptosis-associated morphology (as reviewed in Reed, 2000). Caspases can also cleave members of the Bcl-2 family. For example, anti-apoptotic Bcl-2 can be cleaved by caspase-3 to yield a pro-apoptotic protein (Kirsch *et al.*, 1999).

There are two ways in which caspase cascades can be initiated: the extrinsic pathway in which death receptors are cross-linked by their ligands, and the intrinsic pathway in which apoptogenic factors are released from mitochondria (see figure 8). The binding of a death receptor such as CD95 to its ligand (CD95-L) induces CD95 to trimerize and forma death-inducing complex. This complex recruits procaspase-8 through an adaptor molecule called FADD and activates caspase-8. Caspase-8 cleaves procaspase-3 resulting in its activation and in completion of the apoptotic program. The

second way caspase cascades are initiated is through the release of apoptogenic factors from the mitochondria. Cellular stresses and anti-cancer agents induce the mitochondria to release cytochrome c either through the opening of channels in the outer membrane or through mitochondrial rupture due to organellar swelling as a result of opening of the permeability transition pore. Cytochrome c release intot he cytosol results in the activation of the caspase adaptor Apaf-1 and procaspase-9 which together form a holoenzyme known as the 'apoptosome'. The apoptosome activates caspase-3 and caspase-8 which results in DNA fragmentation and apoptosis (as reviewed by Herr and Debatin, 2001).

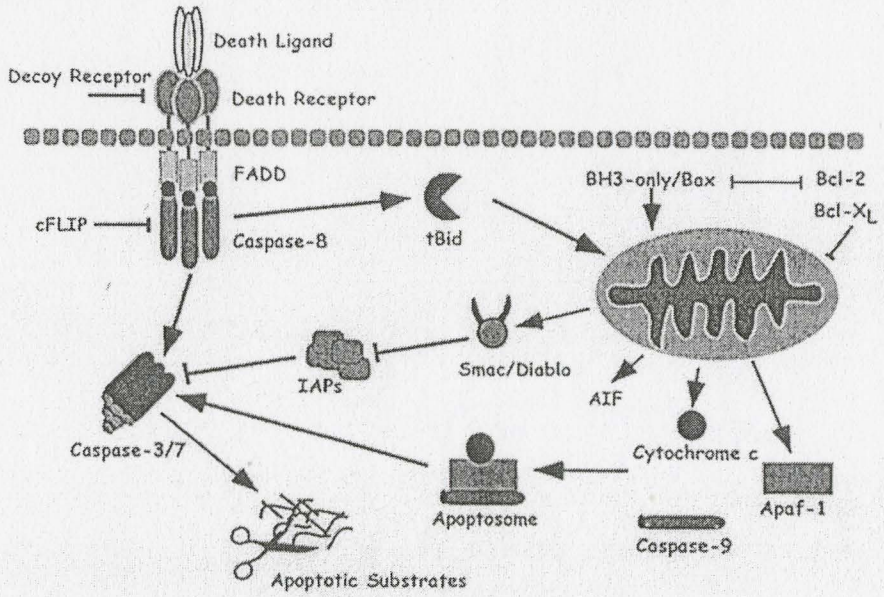


Figure 8: The intrinsic and extrinsic apoptotic pathways (from Herr and Debatin, 2001).

6.1. p53

p53 plays an important role in cellular apoptosis (for reviews see Giacca and Kastan, 1998; Ko and Prives, 1996; Levine, 1997; and Gottlieb and Oren, 1995). In addition, p53 has been shown to play a direct and indirect role in DNA repair (As reviewed by Smith and Seo, 2002; and McKay *et al.*,1999). p53 has a very short half-life of ~5-20 minutes and undergoes ubiquitin-mediated degradation mediated by Mdm-2 (Haupt *et al.*, 1997). Mdm-2 is a negative regulator of p53 by targeting p53 for proteosomal degradation as well as binding to and inhibiting transactivation of p53-responsive genes. In response to many different DNA-damaging agents (ionizing radiation, UV radiation, chemotherapeutic agents), p53 is stabilized and transcriptionally activates or represses several p53-responsive genes. Among the p53 responsive genes is p21 (discussed further below) which activates G1 cell cycle arrest, allowing cells time to repair their DNA damage. However, p53 also transcriptionally activates pro-apoptotic genes such as Bax, and transcriptionally represses anti-apoptotic genes such as Bcl-2 (as reviewed by Weller, 1998). Depending on which way the balance of all of the cumulative signals falls, one of two possible final outcomes results: cell cycle arrest or apoptosis (as reviewed by Schuler and Green, 2001).

The p53 tumour suppressor is the most frequently mutated gene in human cancer (Hollstein *et al.*, 1991) and loss of p53 function may alter cell sensitivity to DNA damage. p53 alterations have been blamed in many cases where cancers have failed to respond to radiotherapy or chemotherapy, and have been a major predictor of response failure (Lowe *et al.*, 1994; and Weller, 1998). In addition, the adenovirus-based transfer

of the wild-type p53 gene into tumour cells which lack p53, has resulted in an increased response of human lung cancer cells to cisplatin (Fujiwara *et al.*, 1994) and in an increased radiosensitivity of ovarian tumour cells (Gallardo *et al.*, 1996).

Mutations in p53 can result in a more stable form of p53 and can decrease sequence-specific DNA binding and transcriptional activity of p53 (as reviewed by Giacca and Kastan, 1998). However, inactivation by mutation is not the sole mechanism of functional p53 disruption, since wild type p53 can be inactivated by interaction with over-expressed Mdm-2 protein (as reviewed by Weller, 1998). Studies have suggested cellular sensitivity to PDT is dependent on functional p53 (Tong *et al.*, 2000, Zhang *et al.*, 1999).

6.2. Bcl2 and Bax

The Bcl-2 family of intracellular proteins is the central regulator of caspase activation in apoptosis (For reviews see Blagosklonny, 2001; Cory and Adams, 2002; and Ruvolo *et al.*, 2001). In mammals, Bcl-2 has at least 20 relatives all of which share at least one conserved Bcl-2 homology (BH) domain (Cory and Adams, 2002). Many Bcl-2 family proteins contain the Bcl-2 homology domains BH1 through BH4. The Bcl-2 family proteins are divided into anti-apoptotic factors: Bcl-2, Bcl-X_L, Bcl-w and CED-9 and the pro-apoptotic factors: Bax, Bak, and the BH3-only subfamily (Bik, Blk, Hrk, BimL, Bad and Bid) (Cory and Adams, 2002). Generally, the anti-apoptotic molecules reside in the mitochondria. Following a variety of death signals, the pro-apoptotic

molecules translocate to the mitochondria as well (Gross *et al.*, 1999; as reviewed in Costantini *et al.*, 2000).

Anti-apoptotic Bcl-2 expression in adults is mostly confined to the long-living cells (eg. stem cell populations, resting B lymphocytes and peripheral neurons) (Maurer *et al.*, 1998). Bcl-2 may prevent apoptosis by blocking cytochrome c release from the mitochondria (Yang *et al.*, 1997; and Kluck *et al.*, 1997), while pro-apototic Bax is believed to contribute to the permeabilization of the mitochondrial membrane allowing for the efflux of apoptogenic proteins (Gross *et al.*, 1999). Bcl-2 heterodimerizes with Bax and flourescently labeled Bcl-2 and Bax proteins have been observed directly interacting in individual mitochondria (Mahajan *et al.*, 1998). It has been proposed that perhaps the Bcl-2 family of proteins multimerize to form pores in the mitochondrial membranes and in that way control the release of mitochondrial apoptotic factors (Reed, 1997). It is thought that the heterodimerization with Bcl-2 is essential for Bax neutralization and cell survival (Oltvai *et al.*, 1993). It is believed that the ratio of Bcl-2 to Bax inside the cell determines whether it will undergo apoptosis after a cytotoxic insult (Oltvai *et al.*, 1993).

It is thought that Bcl-2's function is somehow regulated through its phosphorylation (Ruvolo *et al.*, 2001). The evolutionary conserved Ser^{70} is the critical Bcl-2 phosphorylation site and is thought to be phosphorylated by protein kinase C (PKC) (Ito *et al.*, 1997). The phosphorylation of Bcl-2 probably results in a post-translational modification necessary for it's anti-apoptotic function (Ito *et al.*, 1997).

6.3. BNip3

BNip3 (Bcl-2 and Nineteen kDa interacting protein-3) is a pro-apoptotic, mitochondrial protein which was first isolated through its interaction with the anti-apoptotic adenoviral E1B 19K protein and its functional mammalian homologue Bcl-2 (Boyd *et al.*, 1994). BNip3 overexpression in cells overcomes Bcl-2 (Chen *et al.*, 1997) as well as Bcl-X_L suppression of apoptosis (Yasuda *et al.*, 1998). BNip3 is ubiquitously expressed (Chen *et al.*, 1997; Vande Velde *et al.*, 2000) and hypoxia has been shown to induce up-regulation of BNip3 levels in many types of tumours as well as in endothelial cells and macrophages (Sowter *et al.*, 2001, and Bruick, 2000).

BNip3 has a BH3-like motif between amino acids 110 and 118 resembling the known BH3 domains of various Bcl-2 family proteins (Yasuda *et al.*, 1998, and Chen *et al.*, 1999). The BH3 domain of the pro-apoptotic proteins is essential for their cell death activity as well as for mediating heterodimerization with anti-apoptotic proteins (*Boyd et al.*, 1994, and Wang *et al.*, 1998). Deletion studies of this BH3 domain have suggested that the BH3-like motif of BNip3 is essential for heterodimerization with E1B-19K and Bcl-X_L (Yasuda *et al.*, 1998).

Similar to other Bcl-2 family proteins, BNip3 also contains a C-terminal transmembrane domain (TM domain) that has long been thought to be involved in its homodimerization, pro-apoptotic function, and mitochondrial targeting (Yasuda *et al.*, 1998, and Ray *et al.*, 2000). However, recently Ray and colleagues have shown through deletion studies that the BH3 domain of BNIP3 is actually not involved in BNip3's

heterodimerization with Bcl-2 or Bcl-X_L and the BH3 domain is in fact not responsible for providing its pro-apoptotic activity (Ray *et al.*, 2000). It has been shown that apoptosis induced by BNip3 can be inhibited by both wild type and mutated p53 (175His) (Tsonuda *et al.*, 1999). This supports that there are multiple intracellular pathways that lead to apoptosis and that mutated p53 can still affect BNip3 similar to the wild type p53.

BNip3 has also been shown to contain PEST sequences (Chen *et al.*, 1997). PEST sequences contain high local concentrations of the amino acids P, E, S, T, and D flanked by charged amino acids, and are associated with proteins having a high turnover rate (Rogers *et al.*, 1986). This suggests that BNip3 is a protein susceptible to rapid degradation by proteases and may provide a mechanism for the regulation of intracellular levels of this potentially lethal protein (Chen *et al.*, 1997).

BNip3 migrates as a major band on an SDS-PAGE corresponding to a 60 kDa dimer and as a smaller band corresponding to a 30 kDa monomer even though its apparent molecular weight is actually 21.5 kDa (Chen *et al.*, 1997). Cleavage of the COOH terminus at amino acid 163 results in only the monomer sized band indicating that BNIP3 dimers are formed through the interaction of the COOH terminus of the protein (Chen *et al.*, 1997).

Several sized transcripts of BNip3 exist suggesting that the gene is alternatively spliced or has other closely related genes (Chen *et al.*, 1997). In fact, a human structural and functional BNip3 homologue has been identified called Nix (Chen *et al.*, 1999) (also

known as BNip3∝ (Yasuda *et al.*, 1999)) and an orthologue known as ce BNip3 has been identified in *C. elegans* (Cizeau *et al.*, 2000).

7. The Cell Cycle

The cell cycle is a mechanism by which cells control their division (for review see Sherr, 1993; and Shah and Schwartz, 2001). It is divided into four phases (see figure 9). The first gap phase (G_1) follows mitosis and is a period of growth and metabolic activity. G_1 is followed by the DNA synthesis phase (S) in which the DNA is replicated. The second gap phase (G_2) follows the S phase and precedes the mitosis (M) phase. G_1, S and G_2 are collectively called interphase and certain mature cell types that don't divide remain in interphase in G0 (Weaver, 1999).

The cell cycle is driven by a family of proteins known as cyclin-dependent kinases (CDKs). The transitions between phases are positively regulated by cyclin accumulation and by the activation of cyclin-CDK complexes. They are negatively regulated at the checkpoints by feedback mechanisms such as through CDK phosphorylation, thus preventing the premature entry of the next phase of the cell cycle (Hunter, 1993).

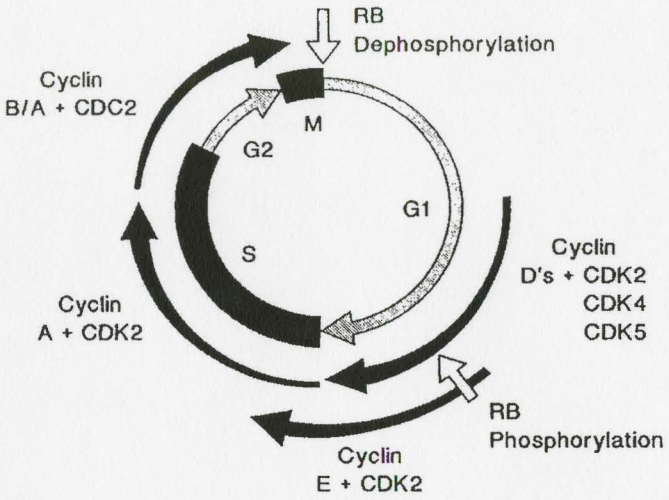


Figure 9· The cell cycle (Sherr, 1993).

7.1. p21

p21 (also known as Waf1, Cip1, or Sid1) is a 21 kDa negative feedback protein which associates with and inhibits the action of multiple CDKs involved in controlling G_1 to S phase transition of the cell cycle (Dulic *et al.*, 1994, El-Deiry *et al.*, 1993, Harper *et al.*, 1993, and Xiong *et al.*, 1993). p21 has specifically been found to immunoprecipitate with cyclin-A, cyclin-D1, cyclin-E and CDK2 (Harper *et al.*, 1993), and has been shown to inhibit the activity of even more members of the cyclin/CDK family (Xiong *et al.*, 1993).

The product (pRB) of the retinoblastoma gene is also responsible for inhibiting cell cycle progression from G_1 to the S phase. Hypophosphorylated pRb binds to E2F which is a known transcription factor, and the pRB/E2F complex binds DNA containing E2F binding sites, repressing transcription of E2F containing genes. Some of the E2F

containing genes are normally expressed in the S phase of the cell cycle. pRB is hyperphosphorylated in the latter part of G_1 by the cyclin/CDKs, causing the pRb/E2F complex to dissociated from the DNA, and allowing for the expression of the E2F containing genes which are necessary for DNA synthesis (as reviewed by Kato *et al.*, 1993; and Sherr, 1993). Thus hyperphosphorylation neutralizes pRb's cell cycle inhibitory properties. Since p21 inhibits the cyclin/CDKs, it is ultimately responsible for preventing the phosphorylation of pRb, thereby arresting cells in G_1.

p21 has been shown to be transcriptionally activated by p53 by direct interaction of p53 with regulatory elements of p21 (El-Deiry *et al.*, 1994). Hence, since DNA damage induces p53, this in turn induces the expresion of p21, which causes the arrest of cells in G1 of the cell cycle, allowing the cells time to repair their damaged DNA before cell division occurs.

p21-mediated growth arrest has also been shown to protect cells against p53 dependent apoptosis (Polyak *et al.*, 1996). p21 expression is inducible by wild type, but not by mutant p53 (El-Deiry *et al.*, 1993). This offers a direct link between a tumour suppressor protein and the cell cycle. The loss of p53 function in tumours results in decreased expression of p21, and an inability of cells to become arrested in G1, causing uncontrolled tumour growth (Hunter, 1993).

8. Heat Shock Proteins

Heat shock proteins are ubiquitously expressed in the cells of all organisms studied (For reviews see Kregel, 2002; and Whitley *et al.*, 1999). They are highly evolutionarily conserved indicating the importance of their intracellular role. The heat shock genes were originally named so because the proteins they code for were at first found to be induced by heat exposure. However, in addition to being induced by heat, it is now known that the heat shock genes are actually induced in response to a wide variety of stimuli, some of which include anoxia, heavy metals, ethanol, nicotine, and viral agents (As reviewed by Whitley *et al.*, 1999). Because heat shock proteins act on a number of different types of stresses, they are often referred to as 'stress' proteins.

Heat shock proteins help rescue cells upon exposure to some kind of non-lethal environmental stress and also help protect cells towards a repeat stress exposure (Lavoie, *et al.*, 1993). Also known as the molecular chaperones, heat shock proteins protect cells by minimizing protein aggregation, assisting in correct folding of polypeptide chains into functional proteins, and by refolding or degrading damaged or denatured proteins. There is an entire family of genes that codes for many different sizes of heat shock proteins and the proteins are categorized into six groups based on their molecular weights. (As reviewed by Kregel, 2002).

8.1. Heat Shock Protein 27

Heat shock protein 27 (Hsp27) is a 27 kDa sized protein and is therefore considered to be one of the small heat shock proteins (As reviewed by Kregel, 2002). Hsp27 was originally identified in the human breast tumor cell line MCF-7 as an estrogen responsive protein (Edwards *et al.*, 1980). Hsp27 exhibits chaperone activity and is involved in modulating actin polymerization, raising intracellular glutathione levels, and inhibiting apoptosis (as reviewed by Concannon *et al.*, 2003).

It has been reported that Hsp27 exists in an equilibrium between small and large oligomers (up to 600-800 kDa large) (Rogalla *et al.*, 1999). Hsp27 phorphorylation at specific serine residues occurs during physiological stress, shifting the equilibrium towards smaller oligomers (Rogalla *et al.*, 1999). After stress induced phosphorylation of Hsp27, it translocates into the nucleus.

Many studies have shown that Hsp27 over expression often correlates with drug resistance and tumour growth in breast cancer and ovarian tumour cells (Huot *et al.*, 1991; Langdon, *et al.*, 1995; Oesterreich *et al.*, 1993; and Yamamoto *et al.*, 2001). Over expression of Hsp27 in some tumour types have been correlated with both a good and a poor prognosis in breast cancer (as reviewed by Ciocca *et al.*, 1993).

9. Project Introduction

Insights into the mechanisms of tumour response and the mode of tumour cell death after PDT may lead to ways that will maximize the efficacy of PDT treatment. Resistant cell lines have been shown to be good model systems in studying the mechanisms of anticancer treatment. It has been previously reported that RIF-1 cells could be made resistant to Photofrin-mediated PDT by repeated exposure to photodynamic treatment with Photofrin (Luna and Gomer, 1991; and Singh *et al.*, 1991). These PDT-resistant cells were further examined for cross-resistance to other agents in order to gain insight into the mechanisms of resistance.

It has been previously reported that three PDT-resistant human colon carcinoma HT29 cell lines were generated and isolated by repeated exposure to PDT using three different photosensitizers (Singh *et al.*, 2001). It was the aim of this study to examine the cross-resistance of these cell lines to other cytotoxic agents including UV radiation and cisplatin. HT29/A11 was generated by 11 cycles of PDT in the presence of aluminum phtalocyanate tetrasulfonate ($AlPcS_4$), HT29/N8 by 8 cycles using Nile Blue A, and HT29/P14 by 14 cycles using Photofrin. The three generated cell lines showed increased resistance to PDT of 1.5 to 2.7-fold (see figure 10) as determined by D_{10} value comparison from clonogenic survival assays using PDT. The photosensitizers used in this study were originally chosen because of their unique intracellular localization sites (Photofrin in the mitochondria, Nile Blue A in the lysosomes, and aluminum phthalocyanine in the plasma membrane). HT29 is a human colon adenocarcinoma that was chosen because colon cancers are clinically treated with PDT because of their ease of

light access, and because any findings towards induced resistance to PDT in HT29 cells would therefore be of clinical relevance (Singh *et al.*, 2001).

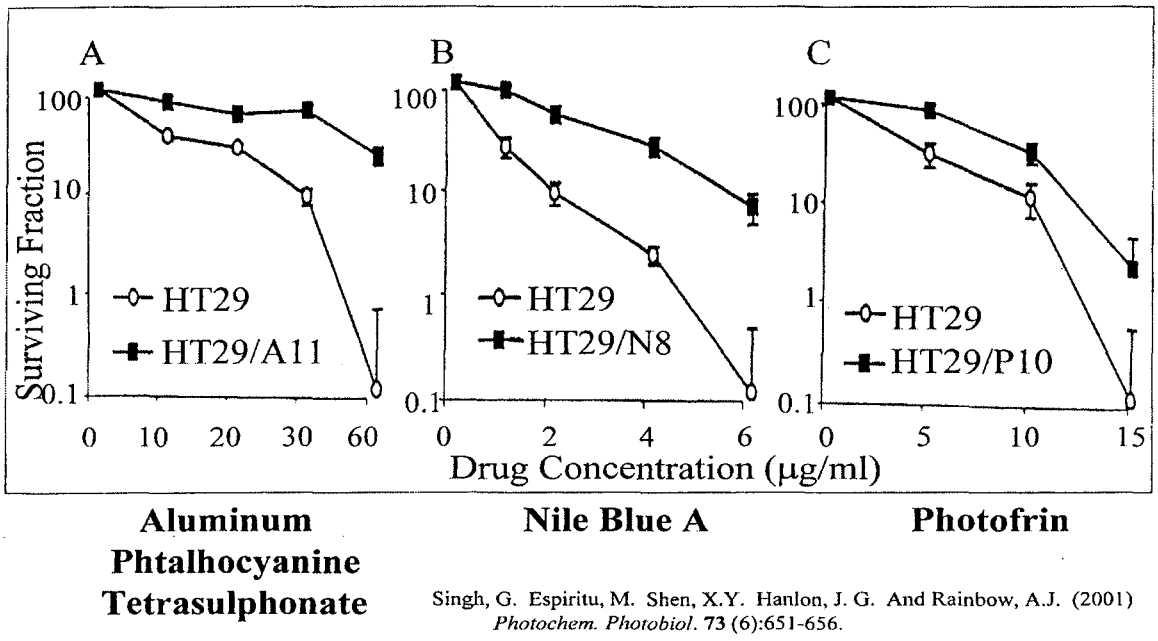


Figure 10: Colonogenic survival curves for HT29 and the PDT-resistant variants. Cells were treated with varying drug concentrations of photosensitizers before exposure to light (525-700 nm light at an energy fluence rate of 9.2 W/m^2. (A) HT29/A11 treated with AlPcS4 (18 h) and then 5.4x10^3 J/m^2 of light, (B) HT29/N8 treated with Nile Blue A (1 h) and then 8.1x10^3 J/m^2, and (C) HT29/P14 treated with Photofrin (18 h) and then 2.7x10^3 J/m^2. Each data point is the average and SE of triplicate determinations in a single experiment. Each experiment was repeated at least three times (from Singh *et al.*, 2001).

It has been previously reported that all three PDT resistant variants show an upregulation of the pro-apoptotic protein BNip3 (Shen *et al.* 2000). The HT29/P14 variant has also been shown to express elevated levels of the heat shock protein 27 (Hsp 27) and overexpression of Hsp27 alone confers HT29 cells resistance to PDT (Wang *et al.* 2002). In addition, all three PDT resistant variants also show increased levels of Bcl-2 but decreased Bax protein expression levels (Shen *et al.* 2000). In the present study we have examined the propterties of these PDT-resistant variants in further detail.

Firstly, the sensitivity of these PDT resistant cell variants to other cytotoxic agents have been examined, specifically UVC, cisplatin, a mixture of UVA and UVB (UVA/B), and UVA alone, by conducting colony survival assays. The cross-reactivity of these cell lines to Photofrin-mediated PDT has also been examined. Protein expression levels of Hsp27, BNip3 and p53 have been examined in these cell lines by quantitating western blot signals, and these have been correlated to cellular sensitivity to UVA, UVC and cisplatin. The DNA repair capabilities of HT29 and of one of the PDT resistant cell lines, HT29/A11, have been examined using HCR assays. The role of p53 in cisplatin senstivity was examined by infecting HT29/P14 cells and parental HT29 cells with adenovirus containing the wild type p53 gene and examining cisplatin sensitivity by colony survival assay. And lastly, preliminary apoptosis assays have been conducted to examine cisplatin-induced apoptosis in HT29/P14 and parental HT29 cells.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Cell lines

HT29 cells were obtained originally from ATCC (Rockville, MD). For the clonogenic assays, two secondary sources of HT29 were used: one being from our own laboratory (HT29a) and the other which was supplied by the HRCC as the cell line used to derive the PDT resistant variants and the Hsp27 and BNIP3 over expressing cell lines (HT29b). The PDT induced variants HT29/A11, HT29/N8 and HT29/P14 (Singh *et al.*, 2001) and the Hsp27 over expressing cell line (HT29/H13) and its control (HT29/Ne015) (Wang *et al.*, 2002) have been described previously. HT29, HT29/A11, HT29/N8 and HT29/P14 cells were grown as cell monolayers and maintained in α-minimum essential medium (α-MEM) containing 10% FBS and 1% antibiotic-antimycotic (all from Gibco-BRL, ON, CA) and kept at 37°C in humidified air containing 5% CO_2. The Hsp27 over expressing cell line (HT29/H13) and its control (HT29/Ne015) were maintained in the same way except that 350 μg/ml of geneticin (G418) (Gibco-BRL, ON, CA) was used in the media instead of the antibiotic-antimycotic to maintain selection pressure on cells containing the neomycin-containing plasmid.

2. Clonogenic Survival Assays

Confluent 75 cm^2 flasks of each cell line were trypsinized, counted with a haemocytometer, and diluted to 100 cells/ml in complete media. 2 ml of cell suspension was plated in each well of a 6-well tissue culture plate to obtain 200 cells per well. Cells were left to adhere for a minimum of 6 hours before treating with cytotoxic agents.

2.1 Cisplatin Treatment of Cells

Cisplatin (in a saline injection - Faulding (Canada) Inc., Cat #1843A) was appropriately diluted in complete growth medium. After the 6 hour adhering period, the growth media was from each well was replaced with 1ml of the appropriate cisplatin dilution, and the plate was incubated for 60 minutes. After incubation, cells were washed in 5 ml PBS, overlayed with 2 ml of cisplatin-free growth media, and further incubated for 6 days to allow for colony growth before staining and counting.

2.2 UV Treatment of Cells

After the 6 hour adhering period, the growth media in each well was replaced with 0.5 ml PBS for UVC and UVA/B irradiation and 1 ml PBS for UVA irradiation. Cells were irradiated for varying times with three different spectra of UV radiation. UVC (254 nm) was generated from a G8T5 (General Electric) germicidal bulb predominantly producing light at 254 nm at a fluence rate of 1 $J/m^2/s$ (as measured by a Blak-Ray J-225 UV meter). UVA/B (19.36% UVB) was generated from a Sciencetech Model 200-1K light source housing a Sciencetech 100-1KMX IKW Hg-Xe lamp at a fluence rate of 4 $J/m^2/s$ (as measured by a Blak-Ray J-221 UV meter). UVA (325-475 nm) (5.62% UVB)

was generated from the same lamp source that creates UVA/B but the UVB was filtered out by a Band-pass filter, and cells were irradiated at a fluence rate of 25 $J/m^2/s$ (as measured by a Black-Ray J-221 long wave UV meter). After irradiation, growth media was added to each well (2 ml for UVC and UVA/B, 4 ml for UVA) and the cells were further incubated for 6 days to allow for colony growth before staining and counting.

2.3 Photodynamic Therapy Treatment of Cells

After the 6 hour adhering period, the growth media from each well was replaced with 7.5 μg/ml Photofrin (QLT Phototherapeutics Inc.) in complete growth medium. This part of the procedure, and all subsequent steps until the cells were stained were done in minimal ambient light conditions. After an 18 hour incubation period, the drug-containing media was removed and replaced with complete growth media and the cells were irradiated for various times with a light bank of fluorescent tubes, filtered with a red acetate filter (Rossolux #19; ROSCO, CA). Three controls were conducted in triplicate for each cell line for each experiment: drug, no light; no drug, no light; and no drug, maximum dose of light given in each experiment. After PDT treatment, cells were incubated for 5 days to allow for colony growth before staining and counting.

2.4 Staining and Counting Colonies

Media was aspirated and colonies were stained with crystal violet (0.5% in 70% ethanol and 10% methanol). Colonies containing at least 20 cells were counted as surviving colonies. Survival was calculated in comparison to mock treated samples. Since there was no significant difference detected between the three controls used for the

PDT experiments (drug, no light; no drug, no light; and no drug, maximum dose of light given in each experiment), the percentage survival values for the PDT experiments were expressed relative to the average of all three controls. Seeding efficiency averaged approximately 50% for each cell line.

3. Host Cell Reactivation Assays

3.1 Virus

Recombinant adenovirus Ad5HCMVsp1*LacZ* was obtained from Dr. F. L. Graham, McMaster University, Hamilton, Ontario, Canada. Ad5HCMVsp1*LacZ* is a nonreplicating recombinant adenovirus expressing β-galactosidase under control of the HCMV immediate early promoter (Morsy *et al.*, 1993).

3.2 Cell Seeding

Cells grown to confluency in 75 cm^2 flasks were trypsinized, counted with a haemocytometer and seeded at 4 x 10^4 cells/well in a 96 well plate. Cells were allowed to adhere overnight before conducting any experiment.

3.3 Ultraviolet Treatment of Cells

When cells in the 96 well plate appeared confluent, the media was aspirated from each well, and 40 μl of PBS was overlayed on each well. 4 vertical rows of the 96 well plate were exposed at any one time to varying doses of UVC (254 nm) from a G8T5 (General Electric) germicidal bulb predominantly producing light at 254 nm at a fluence

rate of 1 J/m^2/s, or UVA/B (19.36% UVB) from a Sciencetech Model 200-1K light source housing a Sciencetech 100-1KMX IKW Hg-Xe lamp at a fluence rate of 20 J/m^2/s (as measured by a Blak-Ray J-221 UV meter). After the irradiation, 160 µl of growth medium was added to each well.

3.4 UV Treatment of Virus

20 µl of Ad5HCMVSp1*LacZ* (Ad) (1.3x10^{10}pfu/ml) was placed in 1.8 ml of ice cold PBS kept in a small (35 x 10 mm) petrie dish on ice. This amount of virus with this protocol results in a multiplicity of infection (MOI) of 10 and is sufficient for the infection of two full 96-well plates of cells. The viral suspension was irradiated using UVC (254 nm) from a G8T5 (General Electric) germicidal bulb predominantly producing light at 254 nm at a fluence rate of 4 J/m^2/s with continuous stirring. At various time points, 200 µl aliquots were sequentially removed and added to 1 ml of serum-free α-MEM such that all UV fluences to the virus in a single experiment were cumulative. The final dose of UVC to virus was always 10,000 J/m^2 in order to completely inactivate all of the virus to be able to determine the background levels of β-galactosidase activity of the cells.

3.5 Viral Infection

One horizontal row was aspirated on the 96 well plate and add 40 µl of appropriate virus sample was added to each well. This was repeated for each row of the 96 well plate and once this was completed, the time was noted to mark the beginning of the infection

period. The plate was incubated for 90 minutes at 37°C in humidified air containing 5% CO_2. After the 90 minute incubation time, 160 µl of growth media was added to each well. The plate was incubated further for 40-44 hours.

3.6 Viral Dilutions for Varying MOI's

The virus was irradiated and prepared as described above except that 80 µl of virus was added to the 1.8 ml of ice cold PBS such that the final infection would result in an MOI of 80. After irradiating, the viral preparations were diluted serially 1:2 to obtain dilutions that would result in MOI's of 40, 20 and 10.

3.7 β-Galactosidase Assay

The media was aspirated from the 96 well plate and 60 µl of CPRG was added to each well. The optical density was taken of each well repetitively over time as a measure of colour change from yellow to purple using a plate reader (Mandel, BIO-TEK Instruments, EL 340 Microplate). OD versus time was plotted in order to observe the saturation curve for one control well and the point taken just below saturation was analyzed in further detail as a survival curve for all treatments. For plotting the survival curves, the average background level of β-galactosidase activity was subtracted from each averaged point from measurements taken from a minimum of triplicate wells.

4. Immunoblot Analysis

4.1 Preparation of Cell Lysates

Confluent monolayers of cells in 100 mm petri dishes were washed in PBS and scraped and suspended in 100 µl of lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 10% protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), pH 8.0) and kept on ice for 60 minutes. The lysate was cleared by centrifugation at 13,000 g for 2 minutes and the protein concentration was determined by the Bradford microassay procedure (BioRad). Lysates were adjusted to a protein concentration of 2 mg/ml in 1x SDS gel loading sample buffer.

4.2 SDS PAGE and Immunoblotting

20 – 40 µg samples were resolved over a 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis for 1 hour at 100 V and either stained with Coomassie Blue stain or transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech) (100 V for 1 hour). Blots were blocked with 20 % skim milk in Tris-buffered saline with Tween 20 (0.05 %) (TBST) for a minimum of 1 hour.

4.3 Immunoblot Analysis

Membranes were incubated for 1 hour at room temperature with either anti-p53 mouse monoclonal IgG (DO-1, Santa Cruz Biotechnology, Inc.) (1:500 in 5 % skim milk in TBST), mouse anti-Hsp27 monoclonal anttbody (SPA-800, Stressgen Biotechnologies) (1:2000), or monoclonal anti- BNip3 from mouse (B7931, Sigma-Aldrich) (1:1000), anti-p21 (sc-187, Santa Cruz Biotechnology, Inc) (1:100). Following primary antibody

incubations, the membranes (except those probed with anti-p53) were washed 3 X in TBST and then incubated with anti-mouse secondary antibody conjugated to horse-radish peroxidase (Santa Cruz Biotechnology, Inc.) (1:5,000) for 1 hour at room temperature. Membranes incubated with anti-P53 were not incubated with a secondary antibody since the anti-P53 was already conjugated to horse-radish peroxidase. Specific antibody-labeled proteins were detected using ECL chemiluminescence detection (Western Lightning, Perkin Elmer Life Sciences, Inc.). Thereafter, blots were stripped (100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, 2% SDS, pH6.7) for 1 hour at 65°C and reprobed with antibodies to actin monoclonal anti-β-actin (A5441, Sigma-Aldrich, Inc.) (1:10,000). The data were analysed using a Kodak Digital Science Image Station 440 CF and protein levels were determined relative to actin levels.

5. p53 and p21 Protein Induction Assays

5.1 p53 Induction Using UVC

Cells were seeded in 100 mm petri dishes and left to adhere to semi-confluency (90%) overnight. Growth media was removed and cells were overlayed with 2mls of PBS, and irradiated with UVC at 1 $J/m^2/s$ for the dose specified. 12 mls of media was added to the cells already containing PBS and the cells were then left in 37°C humidified air containing 5% CO_2 overnight. Cell lysates were prepared for western blotting as described above, 24 hours post cisplatin treatment.

5.2 p21 Induction Using Cisplatin

Cells were seeded in 100 mm petri dishes and left to adhere to semi-confluency (90%) overnight. Growth media was removed and cells were overlayed with 10 mls of media containing the appropriate concentration of cisplatin, and left for one hour in 37°C humidified air containing 5% CO_2. After one hour, the cisplatin-containing media was aspirated and cells were washed with 15 mls PBS and overlayed with 10 ml media. Cells were then left in 37°C humidified air containing 5% CO_2 overnight. Cell lysates were prepared for western blotting 24 hours post cisplatin treatment using the same protocol outlined above.

6. Cisplatin Colony Survival Assays with Overexpression of p53 Using Adenovirus

6.1. p53 Overexpression Using Ad5p53wt

HT29/P14 or parental HT29 cells from three day old semi-confluent cultures were seeded in a twelve well plate at $3X10^5$ cells per well in a 2 ml volume per well. Cells were left overnight in 37°C humidified air containing 5% CO_2. Cells were mock-infected, infected with Ad5p53wt or with AdCA17. Both viruses were obtained from Dr. F. L. Graham, McMaster University, Hamilton, Ontario, Canada. Ad5p53wt is a non-replicating recombinant adenovirus expressing the wild-type p53 gene under the HCMV promoter (Bachetti and Graham, 1993). AdCA17 is a non-replicating adenovirus that contains the β-galactosidase gene under the control of the HCMV promoter (Addison *et al.*, 1997) and was used as the control virus. Infections were done at MOI 100 in a 250 µl volume of α-MEM and left for 90 minutes in 37°C humidified air containing 5% CO_2. After the 90 minute infection incubation, 2 ml of complete media was added to each well to stop the infection. The infected cells were left overnight in 37°C humidified air containing 5% CO_2. Twenty-four hours post-infection, cells were seeded for colony survival at 200 cells/well and treated 6 hours later with cisplatin as described above. Any remaining cells were turned into cell lysates for western blotting. Briefly, the cells suspended in media were centrifuged at 1000 rpm for 10 minutes on the table top centrifuge, washed in 10 ml PBS and centrifuged again, and suspended in 40 µl of lysis buffer. From this point on, the western blotting protocol outlined above was followed.

7. Preliminary Apoptosis Assays

7.1 Apoptosis Assays

Cells were seeded in 100 mm petri dishes or at $3x10^4$ cells per well in a four chamber Lab-TekII chamber slide (Nalge Nunc International) and left to adhere to semi-confluency (90%) overnight. Growth media was removed and cells were overlayed with 10 mls of media containing the appropriate concentration of cisplatin, and left for one hour in 37°C humidified air containing 5% CO_2. After one hour, the cisplatin-containing media was aspirated and cells were washed with 15 mls PBS and overlayed with 10 mls media. Cells were left in 37°C humidified air containing 5% CO_2. Twenty-four or seventy-two hours post cisplatin treatment, apoptosis assays were performed as per the specifications in the *In Situ* Cell Death Detection Kit, AP (Roche, Cat. No. 1 684 809).

Briefly, for the cells grown in LabTek chamber slides, cells were rinsed in PBS, air dried, and the cells on the slides were fixed by placing the slides in 4% paraformaldehyde for one hour. For cells grown in 100mm petrie dishes, cells were scraped into their media, spun down for 10 minutes at 1000 rpms, and rinsed with 10 mls of PBS and spun down again. Cells were suspended in 1 ml of freshly prepared 4% paraformaldehyde in PBS and left to fix for 1 hour at room temperature. 100 µl of each cell suspension was spotted on a microsope slide and left to dry in the chemical fume hood for 1 hour.

After fixation in 4% paraformaldehyde, all slides were rinsed in PBS and placed in cold permeabilisation solution (0.1% Triton X-100, 0.1% Na Citrate) for 2 minutes.

Slides were rinsed twice in PBS and dried around each sample. The tunnel reaction mixture was diluted 1:10 in label solution and applied to each sample (label solution was applied alone as a negative control). Slides were covered with a cover slip and incubated for 60 minutes at 37°C in humidified air in the dark.

Cells grown on Lab-Tek II chamber slides were rinsed twice in PBS and two drops of DAPI Vectashield Mounting Medium for fluorescence with DAPI (Vector Laboratories Inc., Cat# H-1200), was placed on each slide and mounted with a coverslip. Cells were examined under the fluorescence microscope.

Slides containing cells grown in 100 mm petrie dishes were rinsed twice with PBS and 50 µl of converter-AP was applied to each sample. Slides were covered with a cover slip and incubated for 30 minutes at 37°C humidified air in the dark. Slides were rinsed 3 times in PBS and 100 µl of BCIP/NBT (Roche, Cat. No. 1 697 471) substrate solution was applied to each slide, covered with a cover slip and left at room temperature in the dark for 20 minutes. Slides were rinsed 3 times with PBS and a drop of glycerol was applied to each sample and slides were mounted with a cover slip. For each cell sample, four different fields of view were examined under the microscope and stained (apoptotic) cells were counted relative to the number of unstained cells in the same field of view.

RESULTS

RESULTS

1. Sensitivity of Photodynamic Therapy Resistant Human Colon Carcinoma HT29 Cells to UVC, Cisplatin, UVA/B, UVA, and Photofrin-Mediated PDT

Three PDT-resistant HT29 cell lines have been isolated through the repeated exposure of human colon adenocarcinoma HT29 cells to PDT using three different sensitizing agents. HT29/P14 was isolated through repeated exposure to Photofrin (PF)-mediated PDT, HT29/N8 through Nile Blue A (NBA)-mediated PDT, and HT29/A11 though aluminum phthalocyanine tetrasulphonate (AlPcS4)-mediated PDT. Studies have shown that other isolated PDT-resistant cell lines show cross-resistance to other cellular damaging agents (Moorehead *et al.*, 1994). Therefore, it was considered of interest to examine the cellular sensitivity of these PDT-resistant cell lines to other cytotoxic agents, specifically, cisplatin and UV radiation. Cross-resistance to these agents was examined through clonogenic survival assays. In addition, HT29/H13 and HT29/BNIP3, are two cell lines made to stably over express Hsp27 and BNip3, respectively, through transformation with a plasmid containing either the Hsp27 gene (Wang *et al.*, 2002), or the BNip3 gene (Shen *et al.*, 2000). Controls for each of these cell lines were also made by the insertion of an empty vector and these cell lines were named HT29/Ne015 and HT29/PcDNA, respectively. These cell lines were developed since previous results from cDNA microarrays and differential display using HT29/P14 showed that Hsp27 and BNip3 cDNA levels were altered in this cell line compared to their parental HT29 cell

line, suggesting they are involved in Photofrin-mediated PDT resistance. The Hsp27 and BNip3 over expressing cell line and their controls were also examined for cellular sensitivity to cisplatin and UV through clonogenic survival assays, in order to determine if Hsp27 or BNip3 are involved in cellular sensitivity to these agents. The sensitivity of two different sources of HT29 was examined: the HT29 cell line (HT29/Parental) used to derive the PDT-resistant HT29 cell lines, and the BNip3 and Hsp27 over-expressing cell lines and their controls, and an HT29 source from our own lab which was designated HT29a.

Clonogenic survival assays were performed by exposing single cells of each cell line, seeded at 200 cells/well in a six well plate, to varying doses of each cellular damaging agent in triplicate wells, and allowing those cells to form colonies for six days post treatment. A colony was counted as having survived if it contained more than 20 cells. Surviving colonies were plotted as a percentage of the number of colonies counted for the untreated control cells (average of three determinations for each dose to cells ± SE). The dose that resulted in 37% surviving colonies (D_{37}) was determined from each plot of colony survival versus dose and averaged for a number of experiments (a minimum of three for most cell lines). D_{37} was used as a measure of the cellular sensitivity to each agent, such that the higher the average D_{37} for a cell line, the more resistant it was to that particular agent.

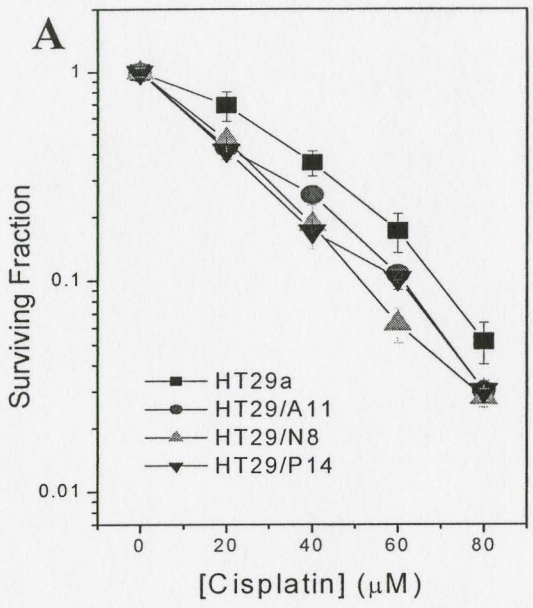
1.1. Cisplatin

It was of interest to examine the cellular sensitivities of the HT29 clonogenic variants in response to cisplatin because cisplatin is thought to exert its cytotoxicity on cells in a different way than PDT does. While PDT causes oxidative damage to cells, cisplatin creates platinum adducts on DNA and proteins. The DNA damage caused by cisplatin is thought to be repaired through the nucleotide excision repair pathway of the cell (Bulmer *et al.*, 1996).

Clonogenic survival assays using cisplatin were performed in at least triplicate for all HT29 clonogenic variants examined, except HT29/P14 and HT29/PcDNA, which were only used in duplicate experiments. Typical results for clonogenic survival assays using cisplatin observed for HT29, the PDT-resistant cell lines and the Hsp27 and BNIp3 over expressing cell lines and their controls, are shown in Figure 1. The average absolute and average D_{37} values relative to HT29 within each experiment are listed in Table 1. For the clonogenic assays using cisplatin, two sources of HT29 were used: one from our own laboratory (HT29a) and the other which was the cell line used to derive the PDT resistant variants and the Hsp27 and BNIP3 over expressing cell lines (HT29/Parental). There was no significant difference between the D_{37}'s obtained from these two sources of HT29 for the clonogenic assays performed using cisplatin indicating that they respond similarly to cisplatin. Therefore the data obtained for the two control HT29 sourced were pooled (HT29-2) (Table 1) and the two cell lines were used interchangeably as controls in individual experiments.

HT29/P14, HT29/A11 and HT29/N8 did not show cross-resistance to cisplatin in the clonogenic survival assays conducted. In fact, all three PDT-resistant cell lines showed a significant ($P < 0.05$) increase in cisplatin sensitivity when assayed by colony survival (Figure 1A, Table 1). The PDT resistant HT29 cell line over expressing Hsp27 alone showed a similar sensitivity to that of parental HT29 cells following cisplatin treatment suggesting that Hsp27 is not involved in HT29 cellular sensitivity to cisplatin (Figure 1B, Table 1). A very surprising result was observed in that HT29/PcDNA showed a 2.35 fold significant ($P < 0.05$) increase in resistance to cisplatin, compared to the HT29 controls (HT29a pooled with HT29/Parental), while HT29/BNIP3 showed no significant difference in sensitivity to cisplatin than the HT29 controls (Figure 1C, Table 1).

Figure 1: Clonogenic survival curves following cisplatin treatment for A. HT29a (■), and the PDT resistant variants HT29/A11 (●), HT29/N8 (▲), and HT/P14(▼); B. HT29/Parental (□), the Hsp27 over expressing cell line HT29/H13 (◆) and its control HT29/Ne015 (◇); and C: HT29/Parental (□), the BNip3 over expressing cell line HT29/BNIP3 (★) and its control HT29/PcDNA (☆). Each data point is the average ± SE of triplicate determinations in a single experiment. Each experiment was repeated at least three times.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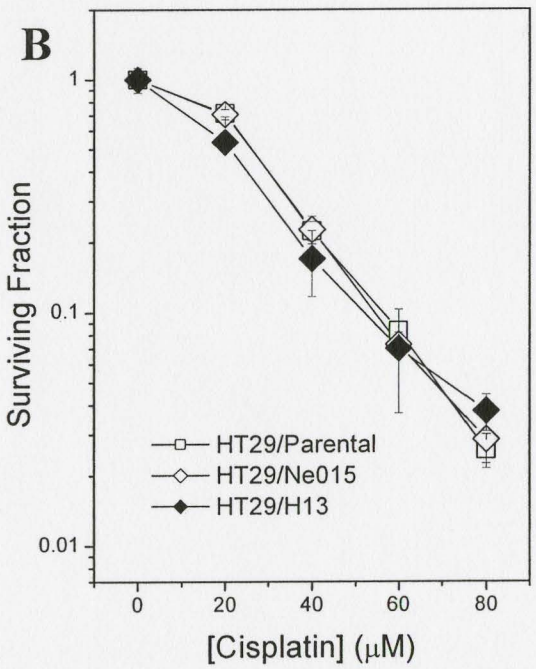
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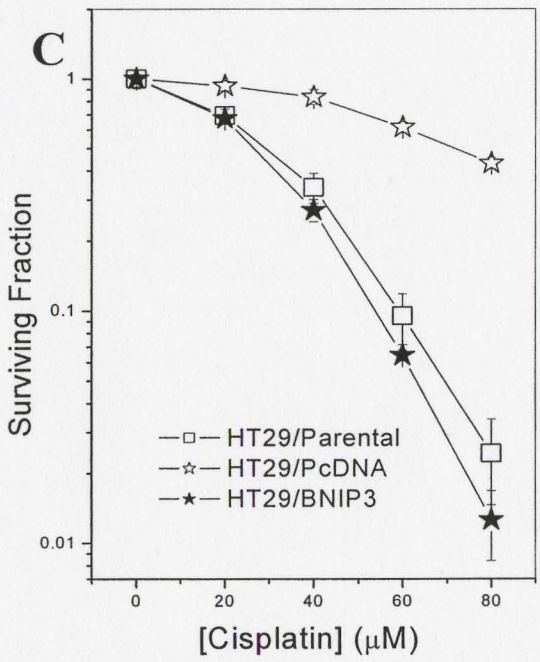
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HT29/PcDNA
HT29/BNIP3

Table 1: The average D_{37} ± SE (μM cisplatin) for colony survival assays with the clonal HT29 variants conducted with cisplatin. "N" represents the number of experiments conducted. Within each experiment the D_{37} for each cell line relative to HT29 was calculated. Some experiments did not have an HT29 cell line as a reference, therefore "n" represents the number of experiments used to obtain that relative D_{37} value. *Significantly different than 1 ($P < 0.05$). Two sources of HT29 were used for these clonogenic assays: one being from our own laboratory (HT29a) and the other which was supplied as the cell line used to derive the PDT resistant variants and the Hsp27 and BNIP3 over expressing cell lines (HT29/Parental). With the use of cisplatin, there was no difference between the D_{37}'s obtained from these two sources of HT29 and therefore, the data obtained for the two sources of HT29 was pooled together (HT29-2).

Cisplatin

Cell lines	N	Absolute D_{37} (μM)	D_{37} Relative to HT29	n
HT29-2	6	40.00 ± 4.63	1	6
HT29/A11	4	24.63 ± 2.05	0.51 ± 0.10 *	3
HT29/N8	4	24.59 ± 0.96	0.68 ± 0.14 *	3
HT29/P14	3	23.95 ± 1.70	0.52 ± 0.05 *	2
HT29/H13	3	45.53 ± 11.06	1.14 ± 0.15	3
HT29/Ne015	3	51.98 ± 12.37	1.31 ± 0.17	3
HT29/BNIP3	3	36.83 ± 5.44	0.96 ± 0.08	3
HT29/PcDNA	2	80.32 ± 7.38	2.35 ± 0 *	2

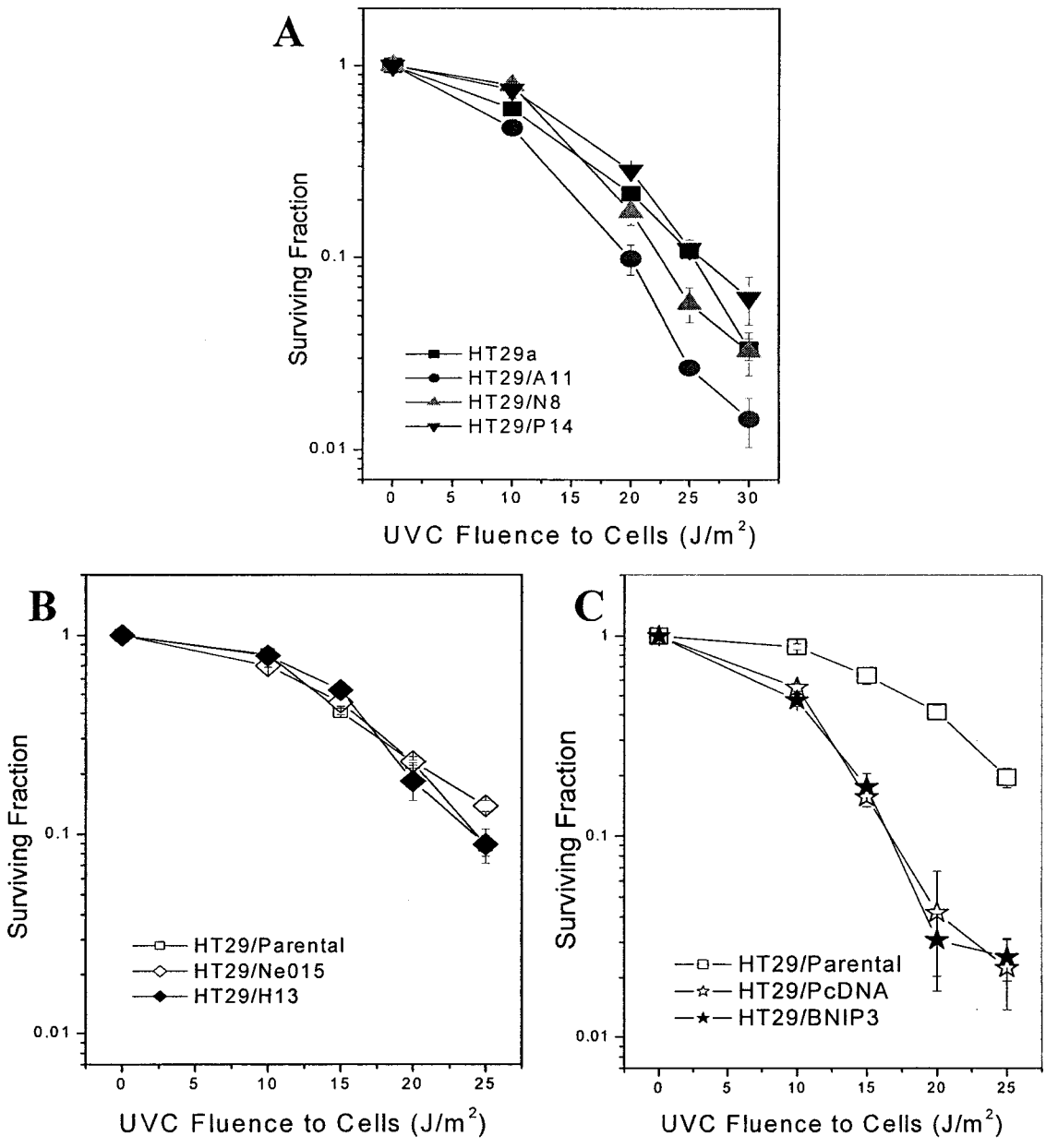
1.2. UVC

Like cisplatin, UVC also induces DNA damage which is repaired by the NER pathway. However, the damage caused by UVC is primarily in the form of cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts (PP), which are different from the platinum adducts caused by cisplatin. Clonogenic survival assays using UVC were performed in at least triplicate experiments for all HT29 clonogenic variants examined. Typical results observed for HT29, the PDT-resistant cell lines and the Hsp27 and BNip3 over expressing cell lines and their controls are shown in Figure 1, and the average D_{37} and average D_{37} relative to HT29 within each experiment are listed in Table 1. As was found for the cisplatin treatment of the two different HT29 cell lines, there was also no significant difference in the D_{37}'s obtained from the HT29/Parental and HT29a cell lines in the clonogenic assays performed using UVC. The data obtained for the two sources of HT29 (HT29/Parental and HT29a) was pooled (HT29-2) (Table 2) and the two cell lines were used interchangeably as controls.

HT29/A11 cells showed a significant increase ($P < 0.05$) in UVC sensitivity, whereas the UVC sensitivity of HT29/N8 and HT29/P14 was not significantly different from that of HT29/Parental cells (Figure 2A, Table 2). A PDT resistant HT29 cell line over expressing Hsp27 alone showed a similar sensitivity to that of parental HT29 cells following UVC treatment suggesting that Hsp27 is not involved in the cellular sensitivity of HT29 cells to UVC (Figure 2B, Table 2). An HT29 cell line over expressing BNIP3 (HT29/BNIP3) and its control (HT29/PcDNA) both showed significant increased ($P <$

0.05) sensitivity to UVC (Figure 2C, Table 2) in comparison to HT29 although HT29/BNIP3 and HT29/PcDNA were not significantly different from each other in cellular sensitivity to UVC.

Figure 2: Clonogenic survival curves following UVC treatment for A: HT29a (■), and the PDT resistant variants HT29/A11 (●), HT29/N8 (▲), and HT/P14(▼); B: HT29/Parental (□), the Hsp27 over expressing cell line HT29/H13 (◆) and its control HT29/Ne015 (◇); and C: HT29/Parental (□), the BNip3 over expressing cell line HT29/BNIP3 (★) and its control HT29/PcDNA (☆) (C). Each data point is the average ± SE of triplicate determinations in a single experiment. Each experiment was repeated at least three times.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HT29/BNIP3
UVC Fluence to Cells (J/m2)

Table 2: The average D_{37} ± SE (J/m^2 UVC) for colony survival assays for the clonal variants of HT29 conducted with UVC. "N" represents the number of experiments conducted. Within each experiment the D_{37} for each cell line relative to HT29 was calculated. Some experiments did not have an HT29 cell line as a reference, therefore "n" represents the number of experiments used to obtain that relative D_{37} value. *Significantly different than 1 ($P < 0.05$). With the use of UVC there was no difference between the D_{37}'s obtained for HT29a and HT29/Parental and therefore, the data obtained for the two sources of HT29 was pooled together (HT29-2).

UVC

Cell lines	N	Absolute D_{37} (J/m^2)	D_{37} Relative to HT29	N
HT29-2	8	15.28 ± 1.45	1	7
HT29/A11	5	11.49 ± 0.56	0.74 ± 0.03 *	4
HT29/N8	5	13.47 ± 0.69	0.95 ± 0.06	4
HT29/P14	4	15.12 ± 1.25	0.99 ± 0.10	3
HT29/H13	3	15.67 ± 0.49	0.87 ± 0.10	3
HT29/Ne015	3	15.71 ± 0.39	0.87 ± 0.09	3
HT29/BNIP3	3	12.12 ± 0.45	0.75 ± 0.14 *	3
HT29/PcDNA	3	11.12 ± 0.42	0.67 ± 0.08 *	3

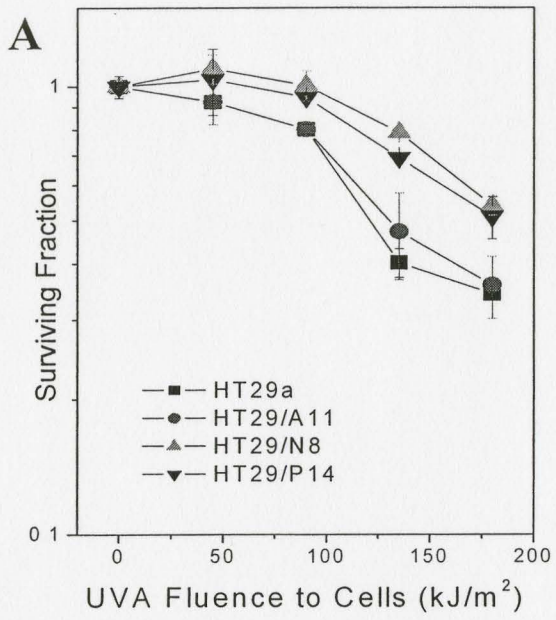
1.3. UVA

UVA like PDT, results in oxidative damage to cells. Therefore, it was of interest to examine the cellular sensitivity of the HT29 clonogenic variants to UVA. Clonogenic survival assays were performed using a UVA source fitted with a Band-pass filter that filters out some of the UVB (5.62% UVB). At least triplicate experiments were performed for all HT29 clonogenic variants examined, except for HT29/Parental, which was only used in one experiment. However, due to the output and beam size limitations of the UVA source, the clonogenic survival assay could only be conducted for one cell line in any one given experiment.

Typical results observed for the clonogenic survival assays for HT29, the PDT-resistant cell lines and the Hsp27 and BNip3 over expressing cell lines and their controls in response to UVA are shown in Figure 3, and the average D_{37} values are listed in Table 3. Relative D_{37} values within a single experiment could not be calculated since only one cell line could be tested in each clonogenic survival assay in any given experiment. Although only one experiment was conducted for the HT29/Parental cell line, it appears that HT29/Parental might be more resistant to UVA than HT29a for which five separate experiments were conducted. This suggests that there might be clonal variability in the reponse of HT29 cells from different sources to UVA. However, more experiments would have to be conducted to determine if there is indeed a significant difference in HT29/Parental and HT29/a cellular sensitivity to UVA.

HT29/P14 and HT29/N8 cells showed a significant ($P < 0.05$) increase in UVA resistance, whereas the UVA sensitivity of HT29/A11 was not significantly different from that of HT29a cells (Figure 3A, Table 3). There was no significant difference in the responses of the Hsp27 over expressing cell line (HT29/H13), its control (HT29/Ne015), or of HT29a cells which do not contain any vector, in cellular sensitivity to UVA (Figure 3B, Table 3). These results suggest that Hsp27 is not involved in cellular response to UVA. The HT29 cell line over expressing BNip3 alone (HT29/BNIP3), its control (HT29/PcDNA), and HT29a cells also showed no difference in cellular sensitivity to UVA (Figures 3C, Table 3), also suggesting that BNip3 is not involved in the cellular response to UVA. There was no significant difference in cellular sensitivity of HT29a, HT29/H13, HT29/Ne015, HT29/BNIP3, and HT29/PcDNA giving further support to the increased resistance of the HT29/P14 and HT29/N8 cell lines to UVA.

Figure 3: Clonogenic survival curves following UVA exposure for A. HT29a (■), and the PDT resistant variants HT29/A11 (●), HT29/N8 (▲), and HT/P14(▼); B. HT29a (■), the Hsp27 over expressing cell line HT29/H13 (◆) and its control HT29/Ne015 (◇); and C: HT29a (■), the BNip3 over expressing cell line HT29/BNIP3 (★) and its control HT29/ PcDNA (☆). Each data point is the average ± SE of duplicate determinations in a single experiment. Each cell line was assayed as a single experiment performed on a separate day and each experiment was repeated at least three times.


A
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Surviving Fraction
HT29a
HT29/A11
HT29/N8
HT29/P14
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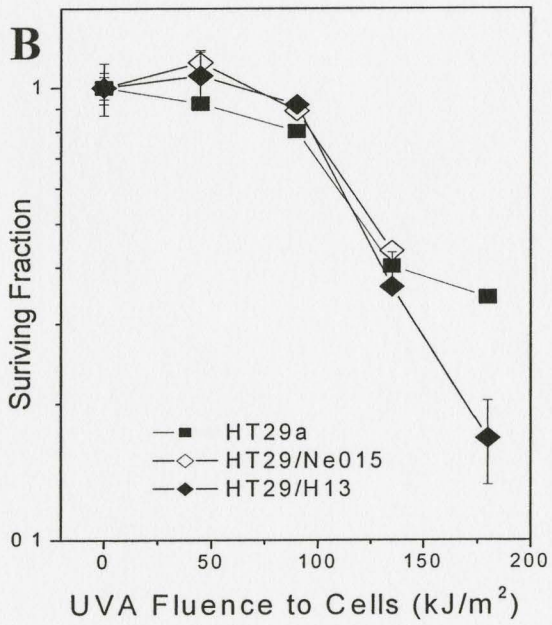
UVA Fluence to Cells (kJ/m^2)


B
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HT29a
HT29/Ne015
HT29/H13
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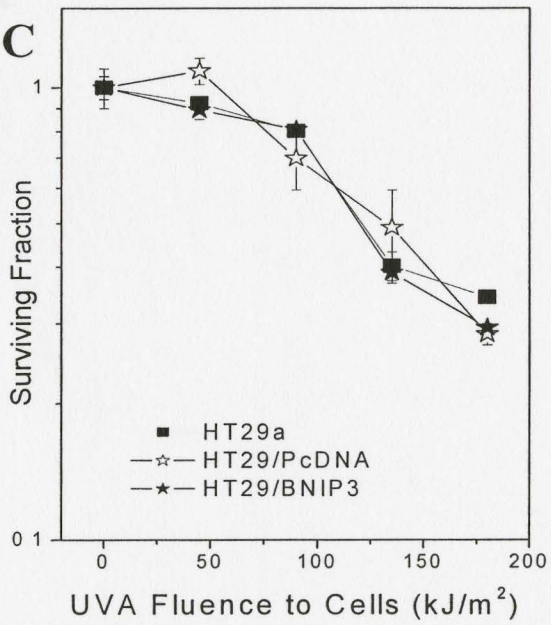
UVA Fluence to Cells (kJ/m^2)


C
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Surviving Fraction
HT29a
HT29/PcDNA
HT29/BNIP3
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UVA Fluence to Cells (kJ/m^2)

Table 3: The average $D_{37} \pm$ SE (J/m^2 UVA) for colony survival assays with the clonal variants of HT29 conducted with UVA. "N" represents the number of experiments conducted. Each colony survival assay for each cell line was conducted on a separate day therefore average relative values are not listed in this table. There was clonal variability between HT29a and the HT29/Parental cell lines tested and their average D37 is therefore calculated separately. * Significantly more resistant to UVA compared to HT29a ($P < 0.05$).

UVA

Cell lines	N	Absolute D_{37} (μM)
HT29a	5	138.3 ± 6.3
HT29/Parental	1	176.4
HT29/A11	4	169.3 ± 30.7
HT29/N8	4	219.05 ± 21.0 *
HT29/P14	4	195.0 ± 13.7 *
HT29/H13	3	130.5 ± 1.9
HT29/Ne015	2	157.0 ± 37.0
HT29/BNIP3	3	127.1 ± 13.4
HT29/PcDNA	3	148.2 ± 10.7

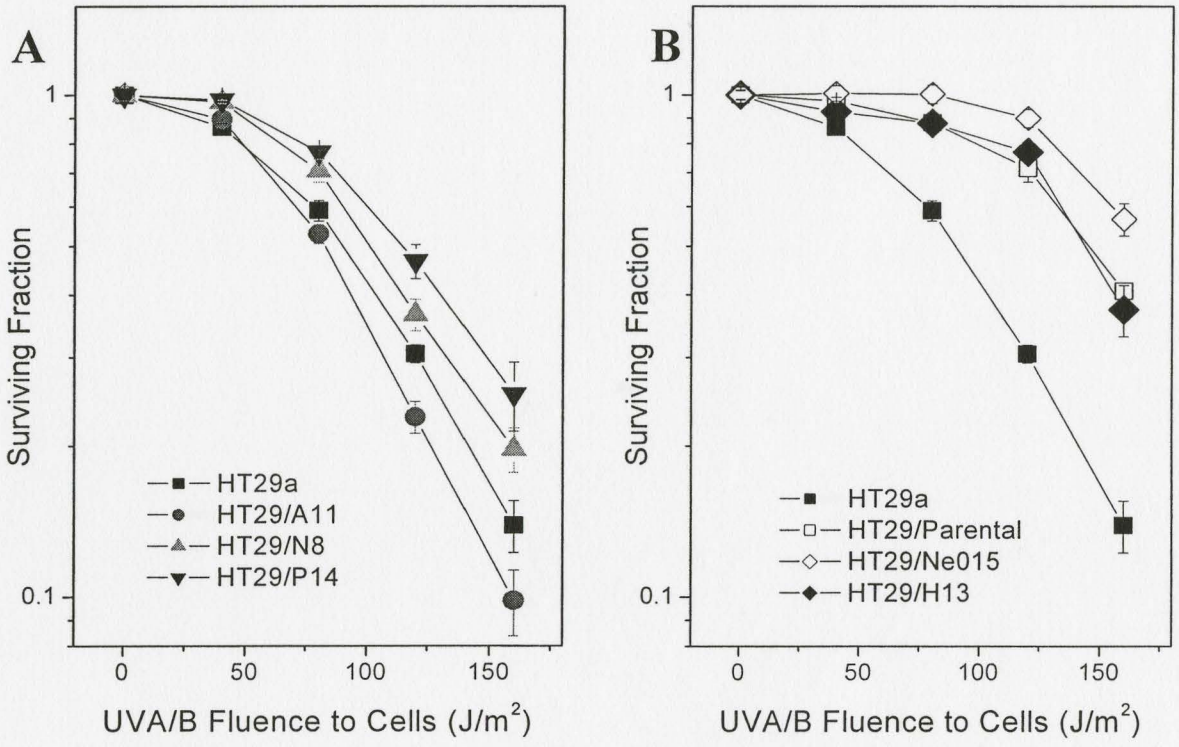
1.4. UVA/B

Like UVA, UVB also causes oxidative damage. However, DNA also absorbs UVB wavelength light directly and therefore UVB causes DNA damage similar to UVC in that it also causes thymine dimers. It was of interest to examine whether the HT29 clonogenic variants respond in the same way to UVA/B as they do to UVC or UVA. Clonogenic survival assays were performed using a UVA source where the Band-pass filter that normally filters out some of the UVB, was removed, such that a mixture of UVA and UVB (~20%) (UVA/B) was emitted. Clonogenic survival assays were performed used UVA/B with only HT29/Parental and HT29a, the three PDT-resistant cell lines, and HT29/H13 and its control, HT29/Ne015. Clonogenic survival assays using UVA/B were performed at least in triplicate experiments for all HT29 clonogenic variants examined except HT29/Parental, which was only used in one experiment, and HT29/Ne015, which was used in duplicate experiments. Typical results for the clonogenic assays of the cell lines tested in response to UVA/B are shown in Figure 4, and the average D_{37} and the average D_{37} relative to HT29a within each experiment are listed in Table 4.

For the clonogenic assays following UVA/B exposure, a difference was detected in the D_{37}s between the HT29a cell line and HT29/H13 and HT29/Ne015 in the first two experiments conducted. Therefore, the experiment was repeated once using the HT29/Parental cells (Figure 4B, Table 4). The HT29/Parental cell line was 1.55 fold more resistant to UVA/B than HT29a in a sole experiment conducted using

HT29/Parental. This suggests that there could be clonal variability in the reponse of HT29 from different sources to UVA/B. However, more experiments would have to be conducted to determine if there is indeed a significant difference in HT29/Parental and HT29/a cellular sensitivity to UVA/B. Another implication of this result is that some PDT-resistant HT29 cell lines might actually be more sensitive to UVA/B than their parental HT29 cell line as was found following UVC exposure. If HT29a is used as the control, then out of the PDT resistant variants, HT29/P14 was the only one that showed a significant increased ($P < 0.05$) resistance to UVA/B, while the other two variants showed no difference (Table 4). However, if HT29/Parental is used as the control, then all of the PDT resistant variants are more sensitive to UVA/B. In comparison to HT29a, HT29/Ne015 and HT29/H13 were both more resistant to UVA/B, with HT29/Ne015 being the most resistant. However compared to HT29/Parental, HT29/H13 and HT29/Ne015 show D_{37} values in comparable ranges.

Figure 4: Clonogenic survival following UVA/B exposure for A. HT29a (■), and the PDT-resistant variants HT29/A11 (●), HT29/N8 (▲), and HT/P14(▼); and B HT29a (■), HT29 parental (□), the Hsp27 overexpressing cell line HT29/H13 (◆) and its control HT29/Ne015 (◇). Cells were treated with varying doses of UVA/B. Each data point is the average ± SE of triplicate determinations in a single experiment. Each experiment was repeated at least three times. This experiment was the only one that included both the HT29/Parental and the HT29a cell lines.


A
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Surviving Fraction
HT29a
HT29/A11
HT29/N8
HT29/P14
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UVA/B Fluence to Cells (J/m2)
B
1
0.1
Surviving Fraction
HT29a
HT29/Parental
HT29/Ne015
HT29/H13
0
50
100
150
UVA/B Fluence to Cells (J/m2)

Figure 5: Clonogenic survival curves following Photofrin-mediated PDT for A. HT29a (■), and HT29/Parental (□); B. HT29/Parental (□) and HT29/A11 (●); C: HT29/Parental (□) and HT29/N8 (▲); and D· HT29/Parental (□) and HT/P14 (▼). Each data point is the average ± SE of duplicate determinations in a single experiment. The control is an average of nine data points, three for each of three conditions: drug, no light; no drug, no light; and no drug, 16 minutes light. There was no significant difference in colony counts for the three control conditions used. Each experiment was repeated at least three times.

Table 4: The average $D_{37} \pm$ SE (J/m^2 UVA/B) for colony survival assays with the clonal variants of HT29 conducted with UVA/B. "N" represents the number of experiments conducted. Two sources of HT29 were used for these clonogenic assays: one being from our own laboratory (HT29) and the other which was supplied as the cell line used to derive the PDT resistant variants and the Hsp27 and BNIP3 over expressing cell lines (HT29/Parental). There was clonal variability between the two different HT29 cell lines tested and their average D37 is therefore calculated separately. Within each experiment the D_{37} for each cell line relative to HT29 was calculated. * Significantly different than 1 ($P < 0.05$).

UVA/B

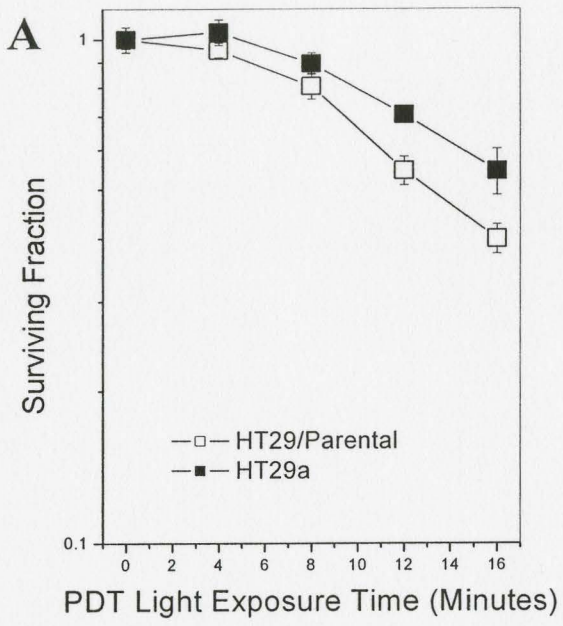
Cell lines	N	Absolute D_{37} (μM)	D_{37} Relative to HT29
HT29a	4	80.40 ± 13.62	1
HT29/Parental	1	167.50	1.55
HT29/A11	4	76.48 ± 7.26	1.02 ± 0.16
HT29/N8	4	95.38 ± 10.36	1.24 ± 0.12
HT29/P14	3	123.13 ± 6.11	1.34 ± 0.05 *
HT29/H13	3	142.23 ± 8.31	1.55 ± 0.05 *
HT29/Ne015	2	176.75 ± 12.62	1.76 ± 0.03 *

1.5. Photofrin-Mediated PDT

The three PDT-resistant HT29 cell lines were isolated through repeated exposure of HT29 to PDT using three different agents: HT29/P14 was isolated through repeated exposure to Photofrin (PF)-mediated PDT, HT29/N8 through Nile Blue A (NBA)-mediated PDT, and HT29/A11 though aluminum phthalocyanine tetrasulphonate (AlPcS4)-mediated PDT. It was therefore of interest to examine whether the two PDT-resistant cell lines isolated using agents different from Photofrin, also show cross-resistance to Photofrin-mediated PDT.

Clonogenic survival assays using Photofrin-mediated PDT were performed at least in triplicate for HT29a, HT29/Parental, and the three PDT-resistant HT29 cell lines isolated through the use of the three different photosensitizing agents (PF, NBA and $AlPcS_4$). Typical results for the clonogenic survival assays using Photofrin-mediated PDT are shown in Figure 5. The average D_{37} relative to HT29/A11 within each experiment is listed in Table 5 for each cell line assayed. HT29/Parental and HT29a showed a difference in sensitivity towards Photofrin-mediated PDT, with HT29a showing a 1.6 fold significantly greater ($P < 0.05$) resistance than HT29/Parental (Figure 5A, Table 5). HT29/A11 and HT29/P14 were both slightly more resistant to Photofrin-mediated PDT than the HT29/Parental cell line (Figures 5B and 5C, Table 5). However, only the increased resistance of HT29/A11 compared to HT29/Parental was significant ($P < 0.05$). These results suggest that the HT29/P14 cell line may have lost its resistance to Photofrin-mediated PDT, or the HT29/Parental cell line may have become more resistant,

both through cellular changes that may have occurred through repeated subculturing for many months. Another explanation is that there was an inability to detect significant PDT resistance in HT29/P14 because clonogenic survival was measured as a function of light exposure for a given Photofrin concentration, rather than as a function of Photofrin concentration for a given light exposure, as was done in the original PDT-resistance experiments in the established HT29 cell lines (Singh *et al.*, 2001). Perhaps our protocol for PDT is not as sensitive in detecting PDT resistance. Out of the three PDT-resistant HT29 cell lines, HT29/N8 appears to be the most sensitive cell line to Photofrin-mediated PDT, and this difference was significant ($P < 0.05$) compared to HT29a as well as HT29/Parental cells (Figure 5D, Table 5).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Surviving Fraction
HT29/Parental
HT29a
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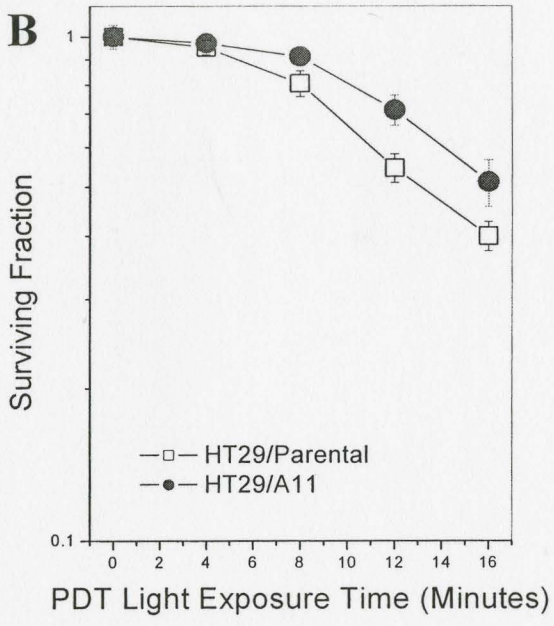
PDT Light Exposure Time (Minutes)


B
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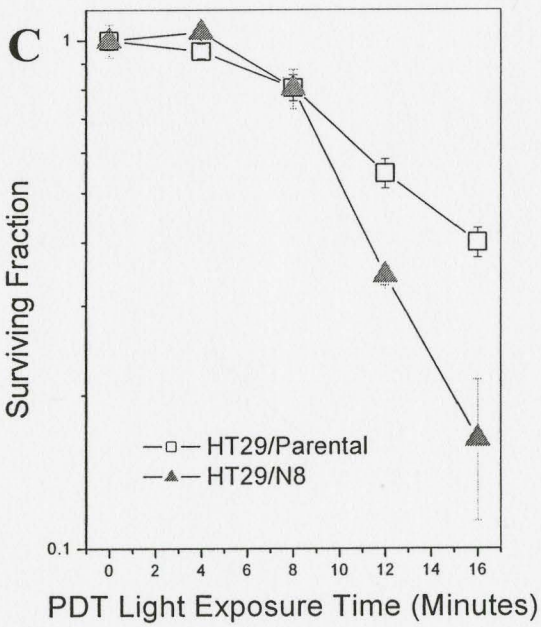
0.1
Surviving Fraction
HT29/Parental
HT29/A11
0 2 4 6 8 10 12 14 16
PDT Light Exposure Time (Minutes)


C
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0.1
Surviving Fraction
HT29/Parental
HT29/N8
0 2 4 6 8 10 12 14 16
PDT Light Exposure Time (Minutes)

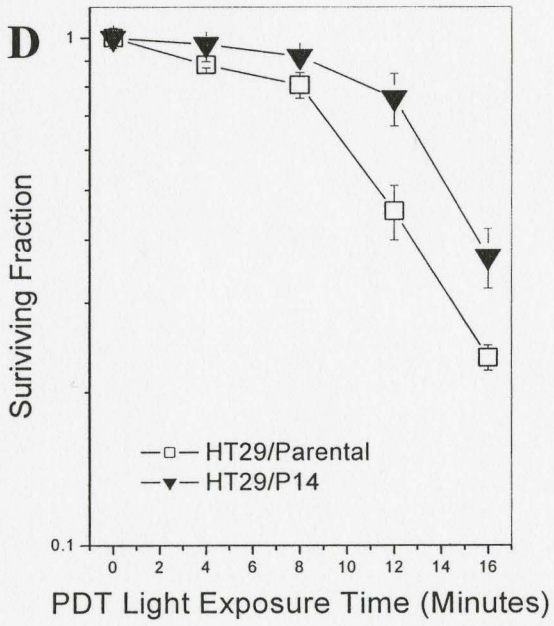

D
1
0.1
Suriviving Fraction
HT29/Parental
HT29/P14
0 2 4 6 8 10 12 14 16
PDT Light Exposure Time (Minutes)

Table 5: The average $D_{37} \pm$ SE (J/m^2) for Photofrin-mediated PDT colony survival assays with the clonal variants of HT29. "N" represents the number of experiments conducted. Two sources of HT29 were used for these clonogenic assays: one being from our own laboratory (HT29) and the other which was supplied as the cell line used to derive the PDT resistant variants and the Hsp27 and BNIP3 over expressing cell lines (HT29/Parental). There was clonal variability between the two different HT29 cell lines tested and their average D37 is therefore calculated separately. Within each experiment the D_{37} for each cell line relative to HT29/A11 was calculated.

Photodynamic Therapy

Cell Lines	N	D_{37} Relative to HT29/A11
HT29	3	1.48 ± 0.27
HT29/Parental	4	0.90 ± 0.04
HT29/A11	5	1 ± 0
HT29/N8	4	0.71 ± 0.04
HT29/P14	4	0.96 ± 0.03

2. Host Cell Reactivation of a UV-Damaged Reporter Gene

Cisplatin induces DNA damage that is repaired by the NER pathway and differences in DNA repair capabilities have been implicated as one of the possible means of cellular resistance to cisplatin (as reviewed by Kartalou and Essigmann, 2001). Host cell reactivation assays using damaged virus containing a β-galacosidase reporter gene, have shown to be useful at providing information about the NER capabilities of cells (Valerie and Singhal, 1995). To examine if there are any differences in NER between HT29 and the most UVC and cisplatin sensitive PDT resistant cell line HT29/A11, HCR assays were performed with Ad5HCMVSP1LacZ virus damaged with UVC. For all viral assays, the HT29a cell line was used as the control cell line.

2.1 Host Cell Reactivation Assays using UVC

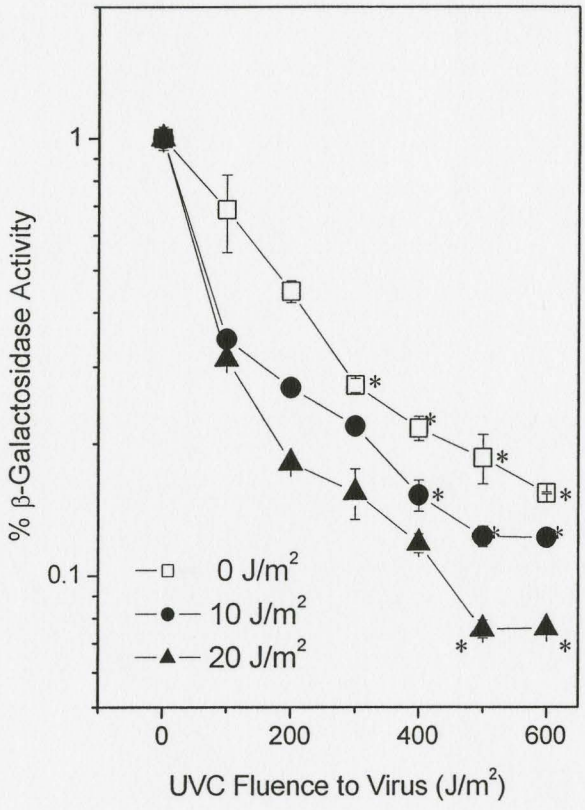
HCR assays were performed on HT29 and HT29/A11 to see if the difference observed in cellular response in the colony survival assays with cisplatin could be attributed to differences in NER abilities. Since HT29/A11 was the most sensitive to both UVC and cisplatin, and both these agents produce DNA damage thought to be repaired through NER, it was of interest to examine if this increased sensitivity to these two agents is the result of a DNA repair deficiency over HT29. For the HCR assays, untreated cells or cells treated with a priming exposure of UVC (10 or 20 J/m^2), were infected in triplicate in a 96 well plate, with Ad5HCMVSp1LacZ virus at MOI 10, which had previously been treated with increasing exposures of UVC radiation or left untreated. The % β-galactosidase activity was plotted as a function of UVC fluence to virus where the % β-galactosidase activity was measured relative to that expressed by cells infected with untreated virus.

Typical results of this HCR assay are shown in Figure 6 and the average exposures to virus resulting in 37% β-galactosidase activity (D_{37}) from four such experiments are shown in Figure 7 and Table 6. It can be seen that HCR of the UV-damaged reporter gene was reduced although insignificantly, in untreated HT29/A11 cells compared to untreated HT29a cells, suggesting a reduced NER of the reporter gene in untreated HT29/A11 cells compared to HT29a cells. In addition, HCR experiments using Ad5HCMVSp1LacZ virus (MOI 10) showed that the dose to virus giving 37% β-galactosidase activity (D_{37}) decreased with increasing UVC dose to HT29a cells (Figure

6A, Table 6, Figure 7). In contrast, for HT29/A11 cells, the 10 J/m^2 UVC dose to cells only showed a slight decrease in D_{37} and the 20 J/m^2 treatment to cells resulted in a UV enhanced reactivation with an increase in D_{37} in three out of the four experiments conducted (Figure 6B, Table 6, Figure 7). The D_{37} for HT29/A11 treated with 20 J/m^2 UVC was significantly greater than that observed for HT29a ($P = 0.04$). The latter result suggests a greater repair of the reporter gene in HT29/A11 compared to HT29a in pretreated (20 J/m^2) cells. Since HT29/A11 and HT29a were infected at the same MOI, it is to be expected that similar amounts of damaged DNA enter both cell types, such that differences in the relative expression of β-galactosidase from the UV-damaged reporter gene reflect reflect differences in cellular repair capacity. However, since HT29a cells are smaller than HT29/A11 cells, it is possible that HT29/A11 and HT29a have different uptake of damaged virus even for the same MOI. Therefore, additional experiments were performed using a range of MOIs.

Figure 6: HT29a (A) and HT29/A11 (B) cells that were or were not irradiated with UVC: 0 J/m^2 (open squares), 10 J/m^2 (closed circles), and 20 J/m^2 (closed triangles), were infected with Ad5HCMVSp1LacZ virus (MOI 10) that had also been irradiated with various doses of UVC (100, 200, 300, 400, 500, 600 J/m^2) or left unirradiated. Cells were incubated in 37)C humidified air and grown for 40 - 44 hours and subsequently assessed for β-galactosidase activity. Each data point is the average ± SE of quadruplicate determinations in a single experiment. Each experiment was repeated four times. * Indicates that a point was not double the background level of β-galactosidase activity of cells treated with completely inactivated virus.

A. HT29a



B. HT29/A11

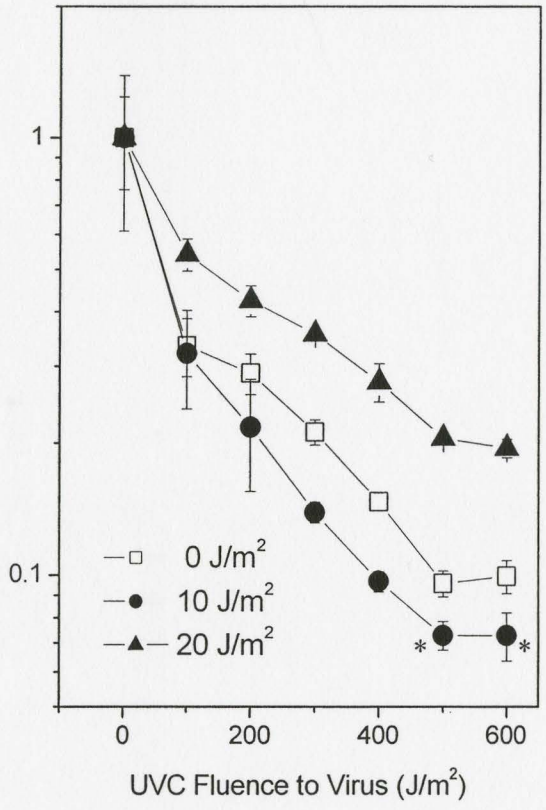
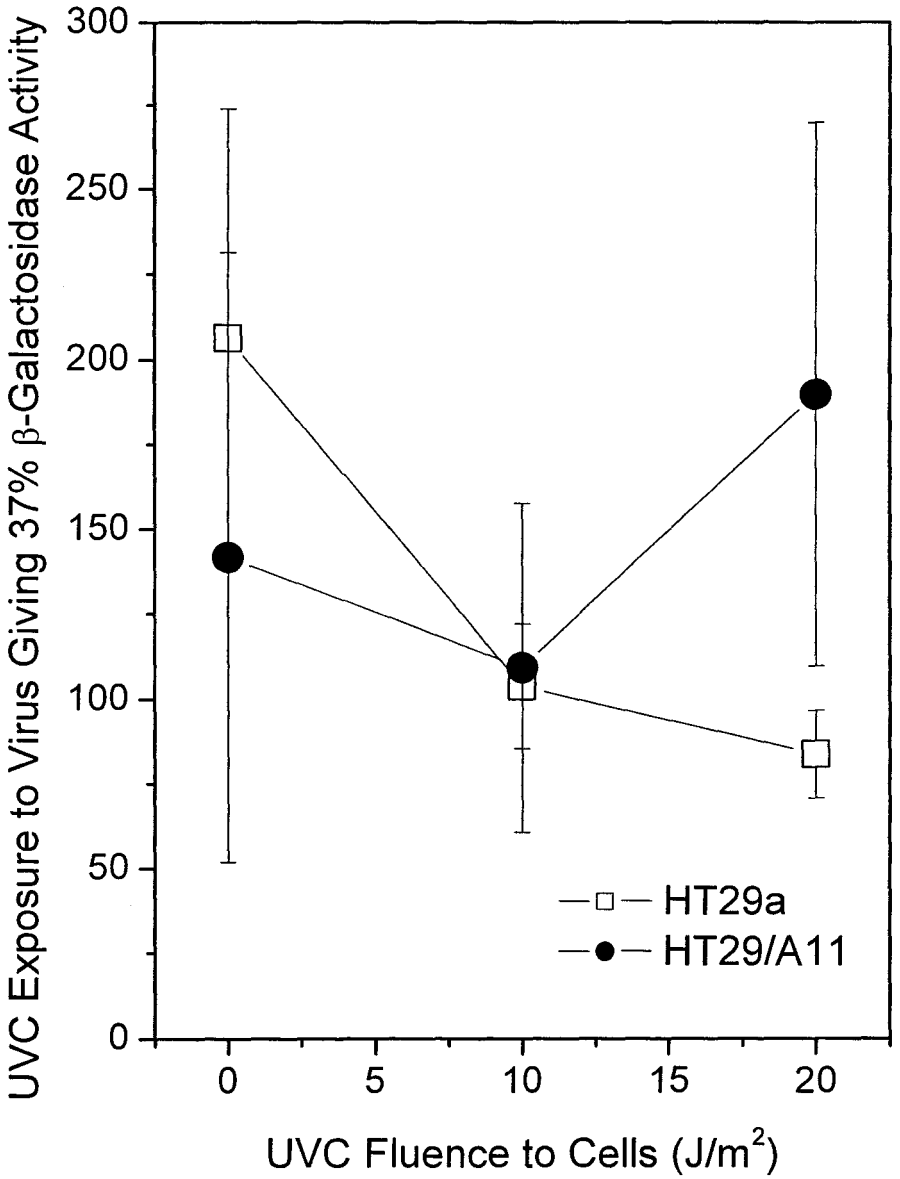


Table 6: A summary of the average UVC exposure to virus giving 37% β-galactosidase activity (± SE) (J/m^2) from four experiments. HT29 and HT29/A11 cells were or were not irradiated with UVC for 0 J/m^2, 10 J/m^2, and 20 J/m^2, and then infected with Ad5HCMVSp1LacZ virus (MOI 10) that had also been irradiated with various doses of UVC (0, 100, 200, 300, 400, 500, 600 J/m^2). Cells were incubated in 37°C humidified air and grown for 40 - 44 hours and subsequently assessed for β-galactosidase activity.

	UVC to Cells	**Average D_{37}**
HT29	0 J/m^2	206.4 ± 33.7
	10 J/m^2	103.7 ± 9.2
	20 J/m^2	83.7 ± 6.5
HT29/A11	0 J/m^2	141.6 ± 44.8
	10 J/m^2	109.2 ± 24.2
	20 J/m^2	189.7 ± 40.0

Figure 7: Average UVC exposure to virus giving 37% β-galactosidase activity (± SD) versus UVC fluence (J/m^2) to HT29a (□), and HT29/A11 (●) cells from four experiments. Cells were or were not irradiated with UVC for 0 J/m^2, 10 J/m^2, and 20 J/m^2, and then infected with Ad5HCMVSp1LacZ virus (MOI 10) that had also been irradiated with various doses of UVC (0, 100, 200, 300, 400, 500, 600 J/m^2). Cells were incubated in 37°C humidified air and grown for 40 - 44 hours and assessed for β-galactosidase activity.


UVC Exposure to Virus Giving 37% β-Galactosidase Activity
300
250
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150
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50
0
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5
10
15
20
UVC Fluence to Cells (J/m²)
HT29a
HT29/A11

2.2 HCR Assays with Varying MOIs of Virus

HCR assays were performed in triplicate with untreated HT29/A11 and HT29a cells infected at varying MOIs with virus irradiated with varying doses of UVC. Typical results are shown in Figure 8. The average dose to virus resulting in 37% β-galactosidase activity (D_{37}) from the three experiments conducted are listed in Table 7. Unirradiated HT29 and HT29/A11 cells consistently showed a decrease in D_{37} with increasing MOI in the three experiments conducted (Figure 8, Table 7). At all MOIs, HT29/A11 had a lower average D_{37} compared to HT29a. There was no significant difference between HT29a and HT29/A11 cells in the average D_{37}s observed for infections conducted at MOIs 10 and 20 (Figure 8, Table 7). However, at MOIs 40 and 80 HT29/A11 showed significantly ($P < 0.05$) lower D_{37}s (Figure 8, Table 7). This suggests that HT29/A11 has a deficiency in DNA repair capabilities over HT29a in untreated cells.

However, the higher MOIs of infection used with HT29a cells (20, 40 and 80) showed lower expression of β-galactosidase activity (data not shown) corresponding to those observed for the lower MOIs of infection used with HT29/A11 (10, 20 and 40, repectively). This indicates that it requires higher MOIs of infection in HT29a cells to attain the same β-galactosidase activity levels as in HT29/A11. This might be due to differences in infection efficiencies because of differences in cellular morphology (HT29a cells are smaller than HT29/A11 cells). However, it is also possible that the infection efficiency is the same in HT29/A11 and HT29, but that expression from the HCMV promoted reporter gene is greater in HT29/A11 cells. Interestingly, it requires double the

HT29a cells compared to HT29/A11 cells, to attain a confluent monolayer of cells, and roughly double the MOI used in infection of HT29a cells provided roughly the same β-galactosidase activity levels as observed for HT29/A11. Therefore, if D_{37}s are compared for HT29a and HT29/A11 where corresponding MOIs resulted in the same β-galactosidase activity for both cell lines, then there appears to be no difference in the DNA damage repair abilities of these two cell lines.

Figure 9 shows results for an HCR experiment using HT29/A11 and HT29a cells that were pre-irradiated with 20 J/m^2 or left untreated and infected with varying MOIs of virus that had been irradiated with varying exposures of UVC. Each cell line was examined on a separate occasion. HCR was lower in unirradiated HT29/A11 cells compared to HT29 at the higher MOIs, similar to that observed in Figure 8. Again, this suggests less repair in HT29/A11 cells when a greater number of damaged lesions are introduced into the cell. For HT29a, pretreatment of cells with UVC lowered the HCR curve for all MOIs of Ad5HCMVSp1LacZ viral infections used (MOIs 10, 20, 40 and 80) (Figure 9A). For the pre-irradiated HT29 cells, there was no difference in the appearance of the HCR curves for all the MOIs used (Figure 9A). For HT29/A11, at MOIs 10 and 20, pre-irradiating the cells resulted in lowering the HCR curves, while at MOI 40, the curves appeared the same for pre- and non-irradiated cells, and at MOI 80 the curve seemed to be higher for the pre-irradiated cells (Figure 9B).

Figure 8: Unirradiated HT29a (A) or HT29/A11 (B) cells were infected with Ad5HCMVSp1LacZ virus at MOI's of 10 (■), 20 (●), 40 (▲), and 80 (▼) and assessed for β-galactosidase activity 40 to 44 hours after the infection was complete. Each data point on the curve is the average ± SE of triplicate determinations. This experiment was performed three times.

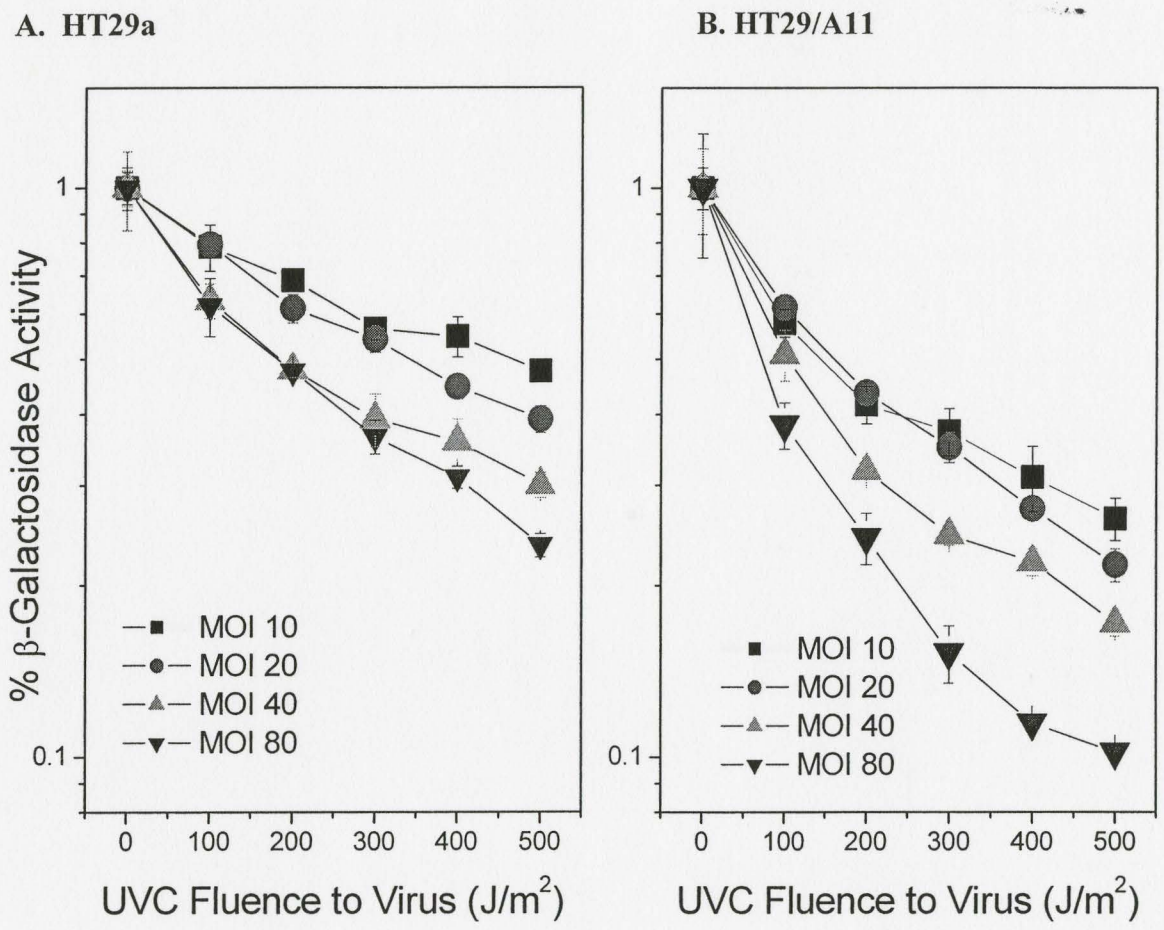
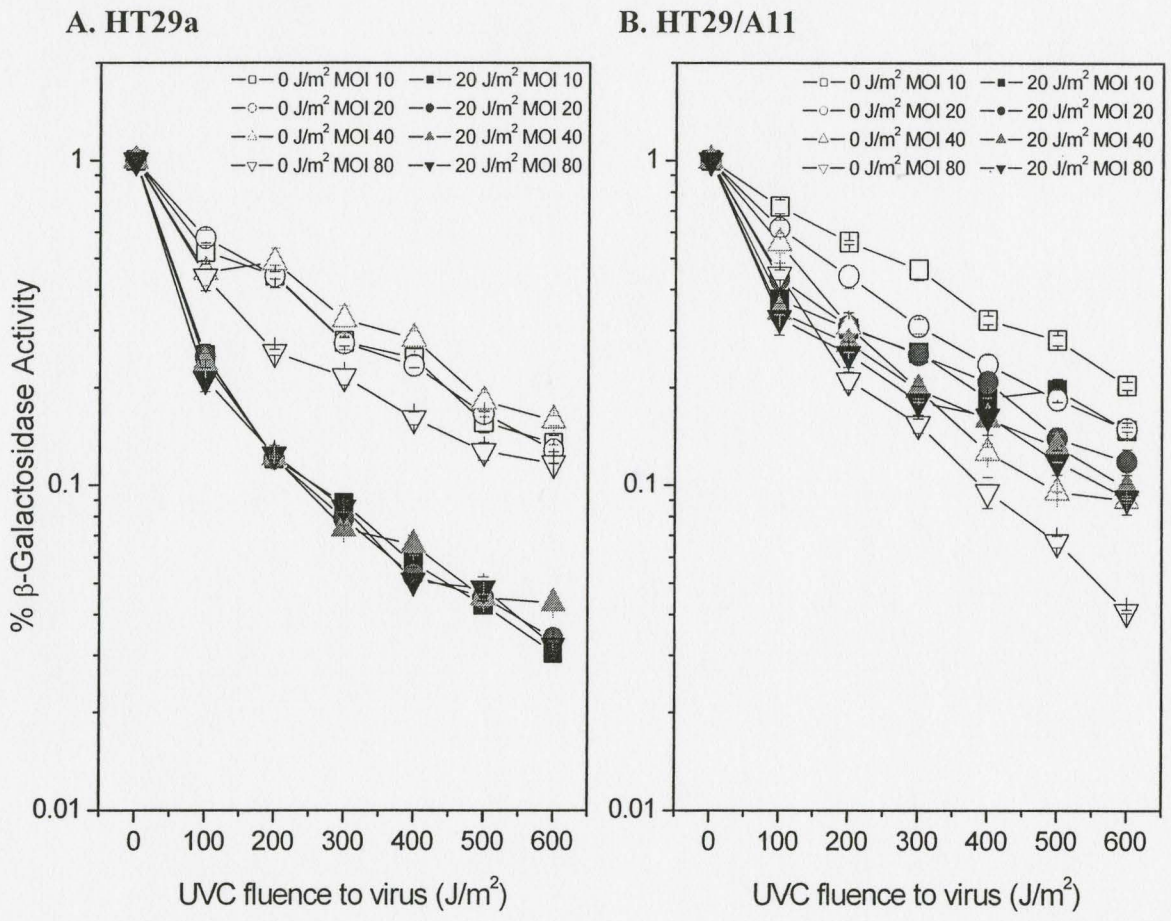

A. HT29a
B. HT29/A11
% β-Galactosidase Activity
1
0.1
0
100
200
300
400
500
MOI 10
MOI 20
MOI 40
MOI 80
UVC Fluence to Virus (J/m²)

Table 7: Summary of the average D_{37} ± SE taken from the HCR survival curves for experiments done with varying MOI's. Unirradiated HT29 or HT29/A11 cells were infected with Ad5HCMVSp1LacZ virus at MOI's of 10, 20, 40, and 80, and assessed for β-galactosidase activity 40 to 44 hours after the infection was complete. The D_{37} was obtained in each experiment where each data point on the survival curve was the average ± SE of triplicate determinations. Each experiment was repeated three times and the average ± SE is listed in the following table.

MOI	HT29	HT29/A11
10	403.5 ± 114.5	316.4 ± 23.4
20	344.0 ± 99.2	301.2 ± 40.0
40	300.9 ± 31.4	168.0 ± 1.1
80	191.1 ± 50.9	104.4 ± 12.9

Figure 9: Unirradiated (open symbols) or 20 J/m^2 UVC irradiated (closed symbols) HT29a (A) or HT29/A11 (B) cells were infected with Ad5HCMVSp1LacZ virus at MOI's of 10 (squares), 20 (circles), 40 (upward triangles), and 80 (downward triangles) and assessed for β-galactosidase activity 40 to 44 hours after the infection was complete. Each data point on the curve is the average ± SE of triplicate determinations. This experiment was only conducted once and each cell line was examined in a separate experiment.


A. HT29a
B. HT29/A11
0 J/m² MOI 10
20 J/m² MOI 10
0 J/m² MOI 20
20 J/m² MOI 20
0 J/m² MOI 40
20 J/m² MOI 40
0 J/m² MOI 80
20 J/m² MOI 80
% β-Galactosidase Activity
1
0.1
0.01
0
100
200
300
400
500
600
UVC fluence to virus (J/m²)

2.3 Host Cell Reactivation Assays using UVA/B

UVA/B causes oxidative damage similar to PDT. It was therefore considered of interest to examine whether HT29a and HT29/A11 or HT29/P14 cells would show any differences in the ability to repair DNA damage induced by UVA/B. If there is a differential ability of the PDT-resistant cell lines to repair DNA damage induced by UVA/B, this might offer some clue as to a possible mechanism of resistance to PDT. HCR assays were performed in triplicate with HT29/A11, HT29/P14 and HT29a cells untreated or pre-treated with 80 J/m^2 UVA/B, and infected at varying MOIs with virus irradiated with varying doses of UVA/B. Results from a single experiment are shown in Figure 10. Similar results were obtained when HCR assays were performed in untreated cells using UVA/B irradiated virus in HT29a and HT29/A11 compared to those obtained for HCR assays using UVC treated virus. Again, a reduced HCR was detected in untreated HT29/A11 and HT29/P14 cells compared to HT29a cells at the higher MOIs, suggesting a reduced repair of UVA/B damage in the HT29/A11 cells. Pretreating cells with UVA/B resulted in a reduced HCR in HT29a cells. No difference was seen in HT29/A11 cells pretreated with UVA/B for MOIs 10, 40 and 80, and a very slight increase in HCR was observed only for MOI 20 in the one experiment conducted (Figure 10). HCR was only slightly reduced in HT29/P14 upon pretreatment with UVA/B (Figure 10).

Upon trying to find MOIs that resulted in equal β-galactosidase expression activity such that D37s could fairly be compared between different cell lines showing

different viral infection efficiencies, there was a secondary complication. There was a change observed over time for HT29/A11 in the β-galactosidase expression rates of the undamaged reporter gene further complicating its comparison to HT29. In three consecutive experiments, HT29/A11 showed increased rates of β-galactosidase expression rates at MOIs 40 and 80, while HT29a β-galactosidase expression rates remained unchanged at the various MOIs examined (Figure 11). This shows that perhaps HT29/A11 was changing over time to become either more easily infected by the virus, or better at protein expression of the infected reporter gene.

Figure 10: Unirradiated (left) and irradiated (80 J/m^2 UVA/B) HT29a (top), HT29/A11 (middle) and HT29/P14 (bottom) cells infected with Ad5HCMVSp1LacZ virus at MOI 10 (■), MOI 20 (●), MOI 40 (▲), and MOI 80 (▼).

HT29a

HT29/A11

HT29/P14

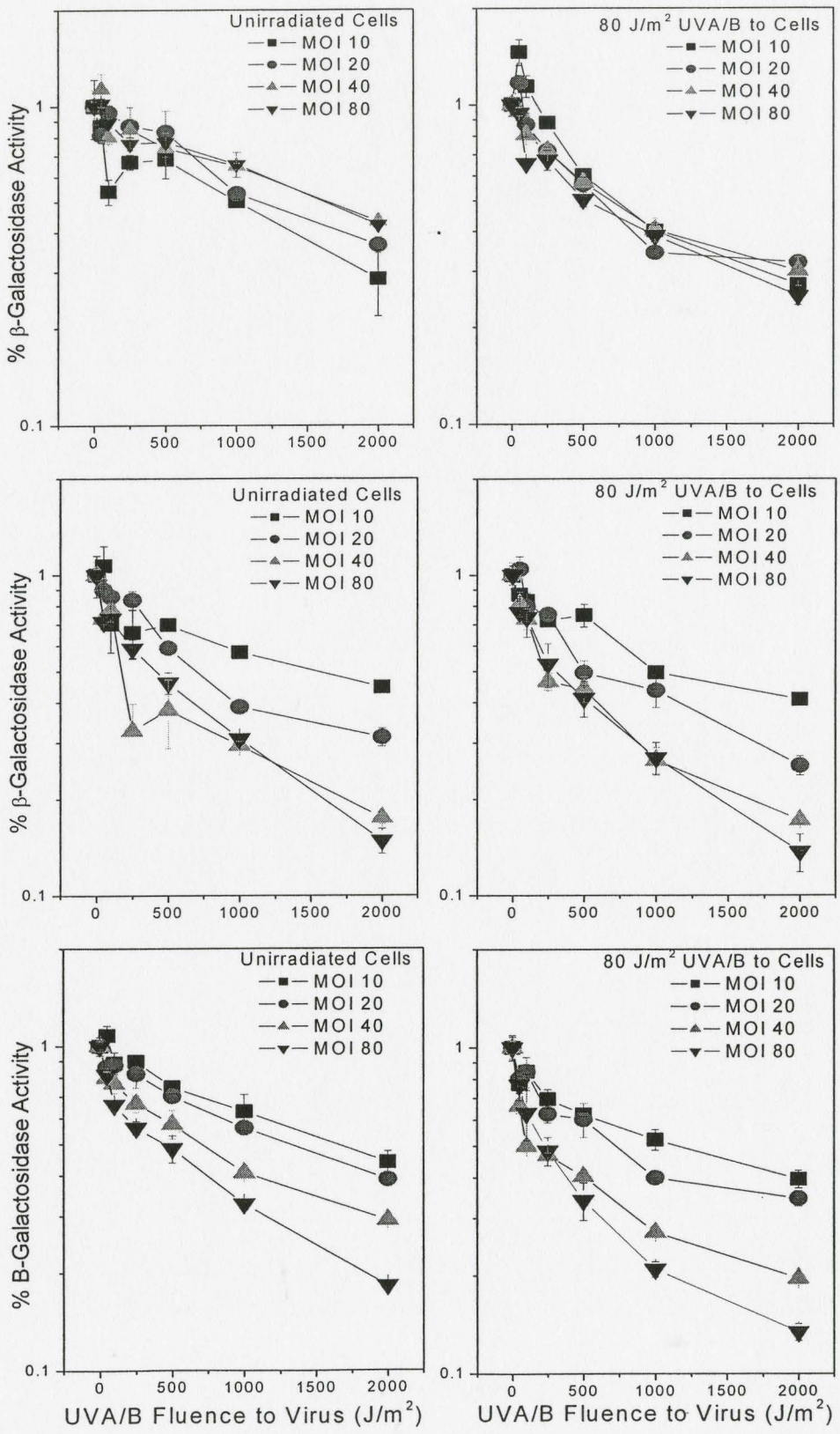
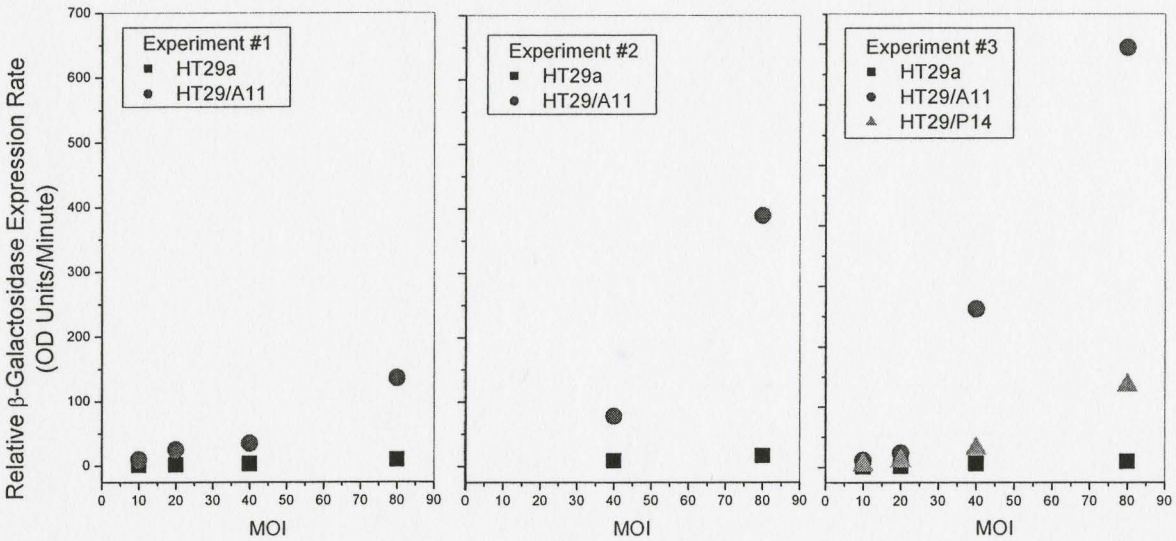


Figure 11: Relative β-galactosidase expression rate (OD units/Minute) plotted as a function of MOI for three separate experiments performed 10 days apart from each other The β-galactosidase expression rate (OD units/Minute) was read as the slope of the linear portion of the saturation curves for the β-galactosidase expression of HT29 (■), HT29/A11 (●), and HT29/P14 (▲) cells treated with undamaged Ad5HCMVSp1LacZ virus.


Relative β-Galactosidase Expression Rate
(OD Units/Minute)
Experiment #1
HT29a
HT29/A11
Experiment #2
HT29a
HT29/A11
Experiment #3
HT29a
HT29/A11
HT29/P14
MOI
MOI
MOI

2.4 Expression of an Undamaged Reporter Gene

It has been previously proposed that UVC treatment to cells results in signals that enhance the transcription and expression of the reporter gene through increased activation of the CMV promoter (Francis and Rainbow, 2000). It has been shown that lower UV exposures to TCR-deficient fibroblast strains, resulted in increased β-galactosidase activity from a CMV promoter, compared to higher UV exposures needed to give the same results in TCR-proficient fibroblast cell strains (Francis and Rainbow, 2000). Since TCR-proficient cell strains repair DNA damage in active genes, these results suggest that it is actually the unrepaired DNA damage in active genes that cause increased reporter activity (Francis and Rainbow, 2000). As a secondary observation from the initial HCR experiments performed in this study, it was observed that with increasing UVC dose to cells treated with the undamaged Ad5HCMVSp1LacZ virus (MOI 10), the β-galactosidase activity also increased. The point in time where β-galactosidase levels for cells infected with unirradiated virus were just pre-saturation for the cells pre-exposed to 20 J/m^2 UVC was chosen. The β-galactosidase levels expressed at that point in time by cells pre-exposed to 10 and 20 J/m^2, were normalized to the control levels expressed by unirradiated cells infected with unirradiated virus at that same point in time within each individual experiment. Pooled results from four experiments are shown in Figure 12 and Table 8 lists a summary of the average normalized β-galactosidase levels. It can be seen that there was no significant difference in UV-induced β-galactosidase expression between the two cell lines suggesting, that there is not a difference in the TCR capabilities of these two cell lines.

Figure 12: A plot of the relative β-galactosidase activity versus UVC exposure to cells (J/m^2) for HT29 (□), and HT29/A11 (●) cells infected with undamaged Ad5HCMVSp1LacZ virus at MOI 10. The point in time where β-galactosidase levels were just pre-saturation for the cells pre-exposed to 20 J/m^2 UVC was chosen, and the β-galactosidase levels expressed by cells pre-exposed to 10 and 20 J/m^2 were normalized to the control levels expressed at that same point in time within each individual experiment. The results of four separate experiments are plotted here.

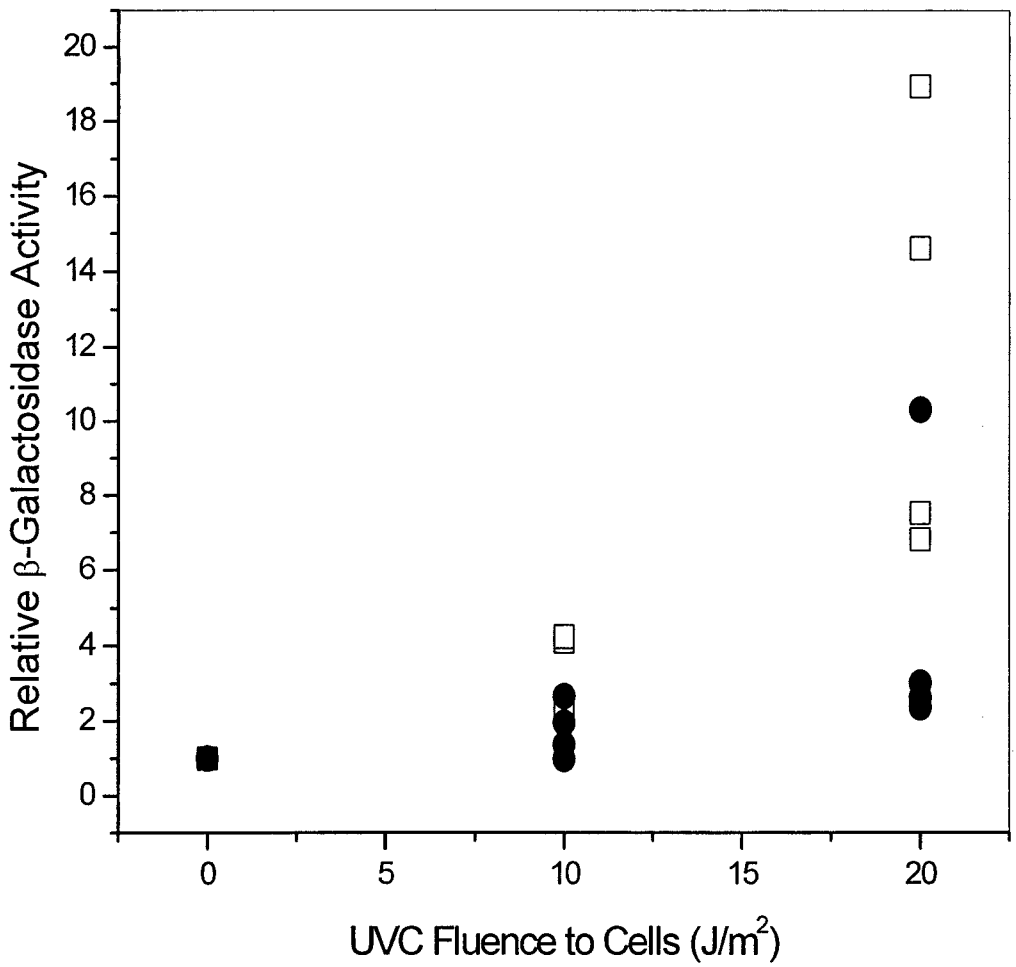

Relative β-Galactosidase Activity
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
UVC Fluence to Cells (J/m²)

Table 8: A summary of the average relative β-galactosidase activities observed for each UVC exposure (J/m^2) to HT29 and HT29/A11 cells infected with untreated Ad5HCMVSp1*LacZ* virus. The point in time where β-galactosidase levels were just pre-saturation for the cells pre-exposed to 20 J/m^2 UVC was chosen, and the β-galactosidase levels expressed by cells pre-exposed to 10 and 20 J/m^2 were normalized to the control levels expressed at that same point in time within each individual experiment. The average (±SD) results of four separate experiments are reported here.

UVC to Cells	HT29	HT29/A11
0 J/m^2	1 ± 0	1 ± 0
10 J/m^2	3.31 ± 1.00	1.74 ± 0.74
20 J/m^2	11.97 ± 5.82	4.58 ± 3.82

3. Protein Expression Levels

Previous experiments with a cDNA microarray and differential display, led to the discovery that Hsp27 and BNip3 protein expression levels are increased in HT29/P14 compared to HT29/Parental cells (Wang *et al.*, 2002; and Shen *et al.*, 2000). Therefore, it was of interest to examine whether the other two PDT-resistant HT29 cell lines also over express Hsp27 or BNip3 protein, despite their isolation using photosensitizers other than Photofrin. Cells from each HT29 clonogenic variant were grown to semi-confluency in 100 mm^2 petri dishes, made into protein lysates, and ran out on SDS-PAGE for western blots. The amount of protein expression was determined by quantifying ECL signals from each western blot, on a Kodak Digital Science Image Station, 440 CF. Protein expression levels were normalized to actin protein expression levels by stripping and re-probing the same blot with anti-actin. The protein/actin expression levels for each cell line was normalized to one cell line, usually HT29a or HT29/Parental. The results from triplicate western blots were averaged.

The more resistant a cell line is to an agent, the greater its D_{37} is observed to be, as read off the survival curve from a clonogenic survival assay. Therefore, 1/ D_{37} is a representation of cellular sensitivity to an agent. All the values for protein expression levels for all of the HT29 clonogenic variants obtained from each individual experiment were plotted against $1/D_{37}$ for each corresponding cell line's response to cisplatin, UVC or UVA and fitted with a regression line. A significant correlation of protein expression

levels to cellular sensitivity to an agent suggests that the protein may be involved in the cellular sensitivity to that agent.

3.1 Hsp27

Typical results for western blots using an antibody probe for Hsp27 are shown in Figure 13A. The average relative Hsp27/Actin protein expression levels to HT29/Parental are shown in Figure 13B. All three PDT-resistant cell lines showed increased Hsp27 protein expression levels as detected by Western blot (Figure 13, A and B). Although HT29/H13 showed twice the Hsp27 protein expression levels than its control (HT29/Ne015), there was no statistically significant difference in Hsp27 protein expression levels between these two cell lines. This suggests that HT29/H13 may be losing its Hsp27 over expression through repeated subculturing. Interestingly, all HT29 cell lines that had been transformed with a plasmid also show increased Hsp27 protein expression levels over the two untransformed HT29 cell lines (HT29a and HT29/Parental), showing that the procedure used to transform cells with plasmid may be stably inducing Hsp27 protein expression. Also, HT29/Parental shows five-fold higher Hsp27 protein expression levels compared to HT29a. If Hsp27 is involved in the cellular response of HT29 to UVA and UVA/B, then the differential expression of Hsp27 in HT29a and HT29/Parental may account for the observed differences in the cellular sensitivities of these two cell lines in response to UVA, and UVA/B. However, plots of Hsp27/Actin protein expression levels attained in all the experiments versus $1/D_{37}$ (used as a measure of sensitivity) for each agent showed no correlation (data not shown) for any

Figure 13: Hsp27 and actin protein expression levels as detected by Western blots in the clonal variants of HT29 (A). Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel. After western blots were probed with anti-Hsp27, they were stripped and re-probed with anti-actin to provide a loading control. Protein amounts were determined by quantifying the ECL signals from Western blots on a Kodak Digital Science Image Station 440 CF. The average (± SE) Hsp27/Actin protein expression levels relative to HT29/Parental were obtained from three separately made lysates (B).

A
HT29a
HT29/A11
HT29/N8
HT29/P14
HT29H13
HT29/Ne015
HT29/BNIP3
HT29/PcDNA
HT29/Parental
Hsp27
Actin
B
Relative Hsp27/Actin Protein Expression Levels
0
5
10
15
20
25
30
35
40
45
50
55
HT29
HT29/A11
HT29/N8
HT29/P14
HT29/H13
HT29/Ne015
HT29/BNIP3
HT29/PcDNA
HT29/Parental

of the agents examined. For cisplatin, R = 0.31 and P = 0.089, for UVC, R = 0.35 and P = 0.056, and for UVA R = - 0.24 and P = 0.191. This suggests that Hsp27 alone is probably not involved in the cellular sensitivity of HT29 cells to cisplatin, UVC or UVA.

3.2 BNip3

Typical results for western blots using an antibody probe for BNip3 are shown in Figure 14A. BNip3 migrates as a major band on an SDS-PAGE corresponding to a 60 kDa dimer and as a smaller band corresponding to a 30 kDa monomer even though its apparent molecular weight is actually 21.5 kDa (Chen *et al.*, 1997). BNip3/Actin protein expression levels were determined only for the 60 kDa band since it was the most intense band out of the three detected for each cell line. The average relative BNip3/Actin protein expression levels to HT29/Parental were determined are shown in Figure 14B. BNip3 protein levels were over expressed in all three PDT-resistant cell lines as well as in HT29/BNIP3 cells that were made to over express BNip3 (Figure 14, A and B).

For all HT29 cell lines that showed over expression of BNip3, a third band even smaller in size than the 30 kDa monomer was detected co-migrating with the 24.7 kDa band of the protein ladder. Perhaps this band corresponds to a degradation product of BNip3 that is detected as a result of degradation during the cell lysate production. Interestingly, the band for HT29/BNIP3 corresponding to this 24.7 kDa sized band is actually shifted up slightly on the western blot. This shift was observed in all three western blots. This shift was also observed in an additional western blot (data not shown) where HT29/BNIP3 was loaded on the gel in the lane right next to the three PDT-resistant

Figure 14: BNip3 and actin protein expression levels as detected by Western blots in the clonal variants of HT29 (A). Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel. After western blots were probed with anti-BNip3, they were stripped and re-probed with anti-actin to provide a loading control. Protein amounts were determined by quantifying the ECL signals from Western blots on a Kodak Digital Science Image Station 440 CF. Only the 60 kDa BNip3 band (which showed the highest intensity) was used for the quantification. The average (± SE) BNip3/Actin protein expression levels relative to HT29 were obtained from three separately made lysates (B).

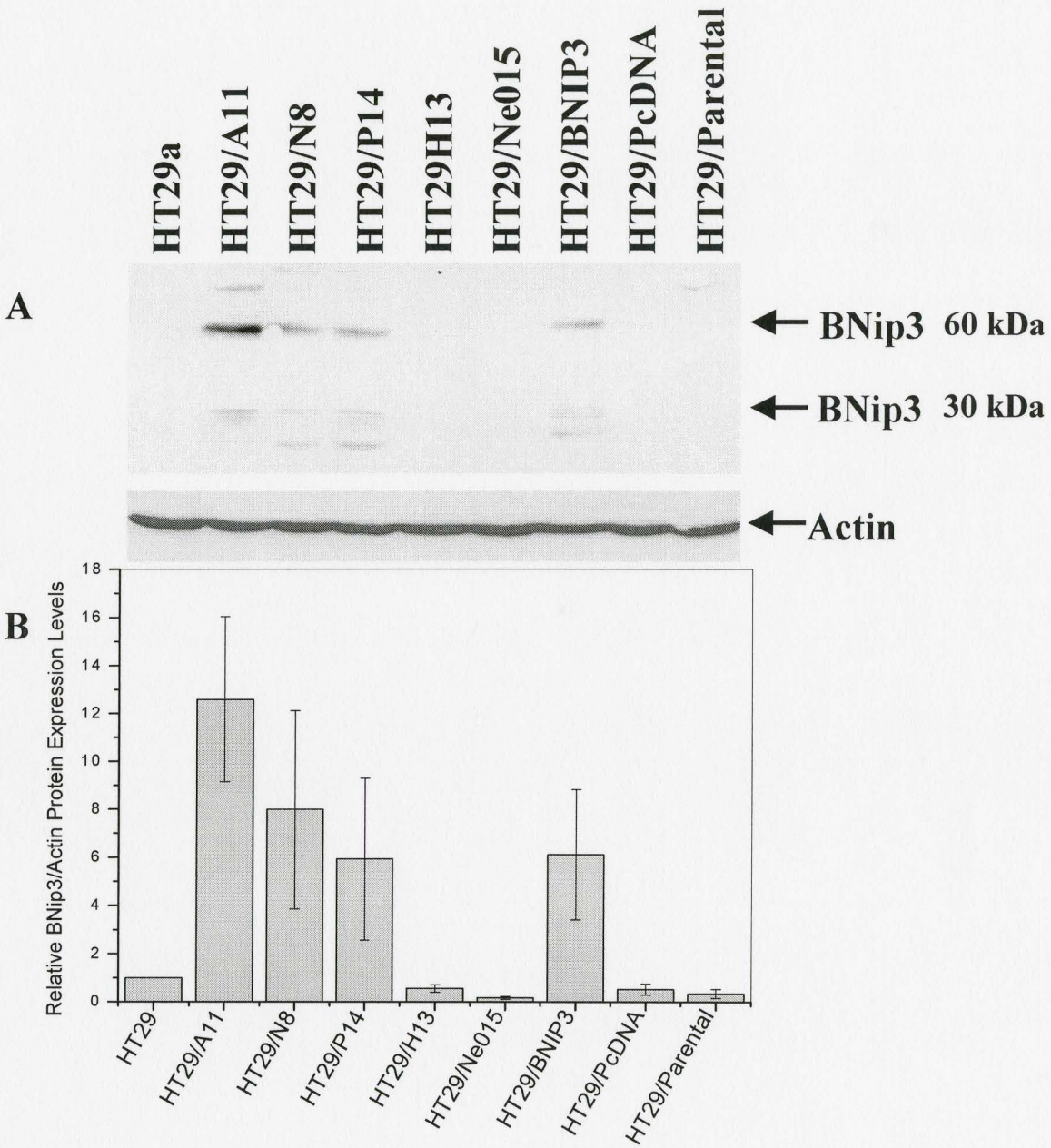
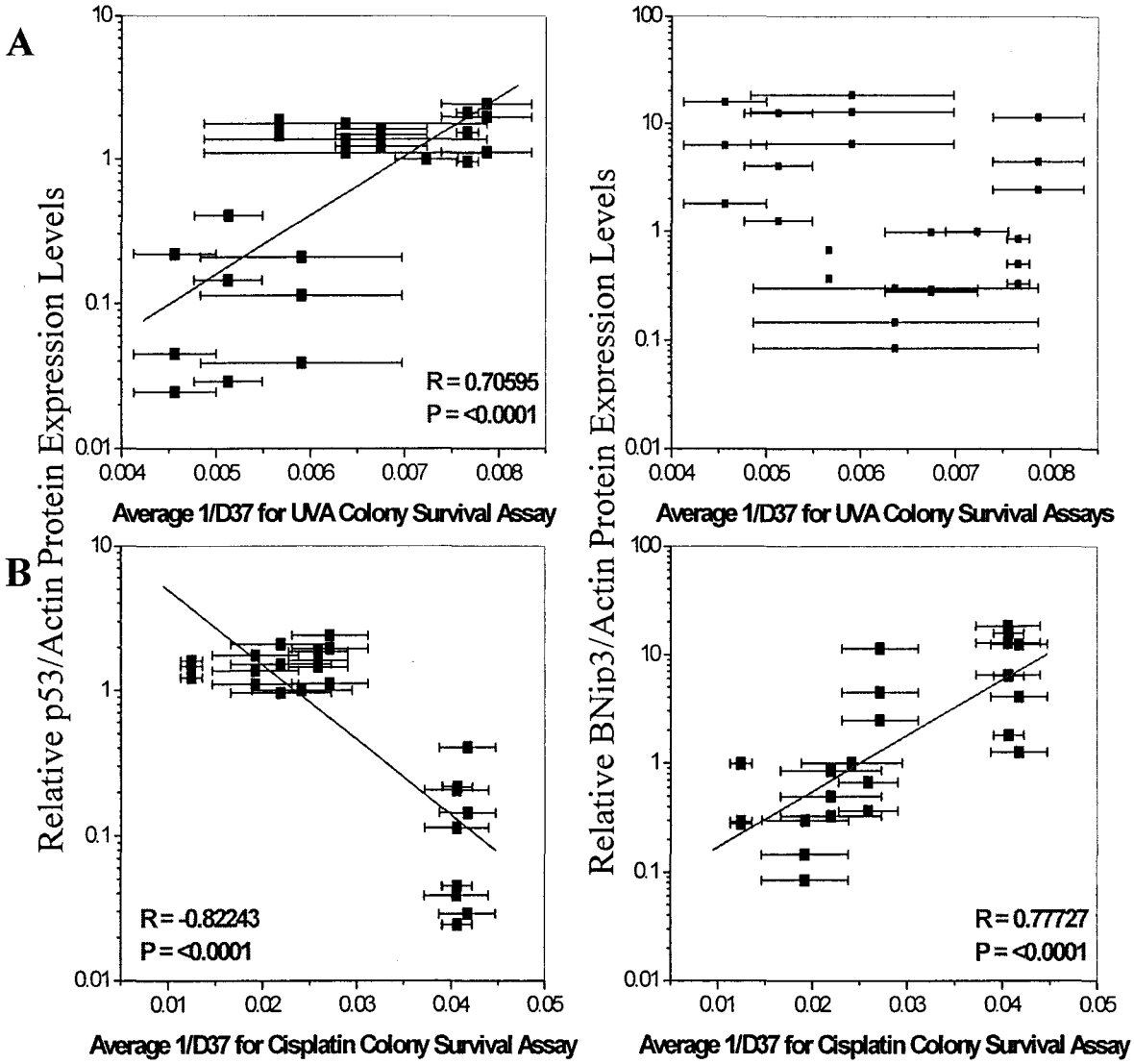

A
HT29a
HT29/A11
HT29/N8
HT29/P14
HT29H13
HT29/Ne015
HT29/BNIP3
HT29/PcDNA
HT29/Parental
BNip3 60 kDa
BNip3 30 kDa
Actin
B
Relative BNip3/Actin Protein Expression Levels
0
2
4
6
8
10
12
14
16
18
HT29
HT29/A11
HT29/N8
HT29/P14
HT29/H13
HT29/Ne015
HT29/BNIP3
HT29/PcDNA
HT29/Parental

Figure 15: Protein/actin expression levels (p53/actin (left), BNip3/actin (right)) relative to HT29 versus 1/D37 (sensitivity) for colony survival assays using UVA (A) and cisplatin (B). Protein expression levels were made by quantitating signals from Western blots using a Kodak Digital Science Image Station 440 CF for three independently made cell lysates for each cell line. These were plotted against the average 1/D37 (± SE) for at least 3 colony survival assay results. Correlation plots include data for three other HT29 clonal variants.


A
B
Relative p53/Actin Protein Expression Levels
Relative BNip3/Actin Protein Expression Levels
R = 0.70595
P = <0.0001
R = -0.82243
P = <0.0001
R = 0.77727
P = <0.0001
Average 1/D37 for UVA Colony Survival Assay
Average 1/D37 for UVA Colony Survival Assays
Average 1/D37 for Cisplatin Colony Survival Assay
Average 1/D37 for Cisplatin Colony Survival Assay

cell lines, to ensure that this observation was not an artifact of how the gel was run. This implies that the BNip3 overexpressed in the HT29/BNIP3 cell line is slightly different than that expressed in the three PDT-resistant cell lines.

In addition, BNip3 protein expression levels showed a positive correlation to cisplatin sensitivity ($R = 0.777$, $P = <0.0001$) (Figure 15B; right panel), and a slightly positive correlation to UVC sensitivity ($R = 0.417$, $P = 0.034$) (data not shown). However, BNip3 protein expression levels showed a sightly negative but insignificant correlation to cell sensitivity to UVA ($R = -0.308$, $P = 0.125$) (Figure 15A; right panel). Since the more BNip3 protein a cell line expresses, the more sensitive it is to cisplatin and UVC, this suggests that the increased cellular expression of BNip3 in the PDT-resistant cell lines, makes them more sensitive to cisplatin and UVC. The lack of a correlation observed for BNip3 expression with sensitivity to UVA, suggests BNip3 is not involved in cellular sensitivity to UVA.

3.3 p53

Since differences in BNip3 protein expression levels were observed in the PDT-resistant cell lines and the HT29 control cell lines, and BNip3 is a pro-apoptotic factor, it was of interest to examine the p53 protein expression levels in these cell lines as well since p53 is also involved in apoptosis. Although HT29 has a mutant p53, it is still recognized by the anti-p53 antibody used in these experiments. Typical results for western blots using an antibody probe for p53 is shown in Figure 16A. In contrast to the

Figure 16: p53 and actin protein expression levels as detected by Western blots in the clonal variants of HT29 (A). Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel. After western blots were probed with anti-p53, they were stripped and re-probed with anti-actin to provide a loading control. Protein amounts were determined by quantifying the ECL signals from Western blots on a Kodak Digital Science Image Station 440 CF. The average (± SE) p53/Actin protein expression levels relative to HT29/Parental were obtained from three separately made lysates (B).

A
HT29a
HT29/A11
HT29/N8
HT29/P14
HT29H13
HT29/Ne015
HT29/BNIP3
HT29/PcDNA
HT29/Parental
p53
Actin
B
Relative p53/Actin Protein Expression Levels
0
2
4
6
8
10
12
14
16
18
20
22
24
HT29
HT29/A11
HT29/N8
HT29/P14
HT29/H13
HT29/Ne015
HT29/BNIP3
HT29/PcDNA
HT29/Parental

increase in BNip3 protein expression levels, all three PDT-resistant cell lines showed a down regulation of p53 protein expression compared to parental HT29 (Figure 16, A and B). Also of note is that the HT29/Parental cell line showed a significantly ($P = 0.005$) slightly higher (1.7 fold) protein expression level of p53 than HT29a.

p53 protein expression levels showed a positive correlation to UVA sensitivity, where $1/D_{37}$ was used as a measure of cell sensitivity ($R = 0.706$, $P = <0.0001$) (Figure 15A; left panel), a negative correlation to cisplatin sensitivity ($R = -0.822$, $P = <0/0001$) (Figure 15B; left panel), but no correlation to UVC sensitivity ($R = -0.212$, $P = 0.289$) (data not shown). This suggests that the higher the p53 protein expression levels in a cell, the more sensitive they are to UVA, whereas the more resistant they are to cisplatin.

Correlations were examined for different ratios of each protein to determine if there was any particular combination of these three proteins that is important for cellular sensitivity to these agents. The best correlation was obtained from a plot of BNip3/p53 versus cisplatin sensitivity ($R = 0.89$, $P < 0.0001$) (data not shown) while the correlation of BNip3/p53 to survival after UVA was actually worse ($R = -0.57$, $P = 0.002$) (data not shown) than for the plot of p53 protein alone. This suggests that the ratio of BNip3 to p53 protein expression is an important determinant of cell sensitivity to cisplatin. The correlation results are summarized in Table 9.

Table 9: A summary of the correlations between protein expression levels and sensitivities of the HT29 clonal variants to the various agents examined in colony survival assays. 0 = no relationship. + = significant ($P < 0.05$) positive correlation: higher protein expression levels resulted in higher sensitivity to the agent. - = significant ($P < 0.05$) negative correlation: lower protein expression levels resulted in higher sensitivity to the agent.

	UVC	Cisplatin	UVA
BNip3	+	+	0
p53	0	-	+
Hsp27	0	0	0

4. p53 and p21 Protein Induction Assays Using UVC and Cisplatin

The greater cisplatin resistance of HT29 cells compared to the PDT-resistant variants could result from a functioning p53 in HT29 cells which can upregulate p21 resulting in cell cycle arrest and increased DNA repair after cisplatin treatment. Although HT29 expresses a mutant p53, it may have some function. It was therefore considered of interest to examine the regulation of p53 and p21 in HT29 and the PDT resistant variants following exposure to UVC and cisplatin.

4.1 p53 Induction Assays Using UVC

p53 protein expression levels are known to be induced in cells in response to UVC radiation and therefore UVC radiation was used to test p53 induction in these cell lines. HT29/Parental, HT29/A11, HT29/P14 and HCT/116 cells allowed to grow to semi-confluency were treated with 30 J/m^2 UVC and made into cell lysates 24 hours post UVC treatment. The lysates were run out for western blots and probed with anti-p53. p53 protein expression levels were compared to actin levels in the same blot by stripping and re-probing the blot with anti-actin. The HCT/116 cells were used as a positive control because it has a wild-type p53, known to be inducible by UVC radiation. 30 J/m^2 was the UVC fluence amount chosen for the exposure since it was the highest exposure used in the UVC clonogenic survival assays and was therefore known to have some effect on the HT29 clonogenic variants.

These p53 induction experiments were performed in duplicate and typical results for HT29/A11 are shown in Figure 17 and for HT29/P14 in Figure 18. p53 levels could not be shown by western blot to be inducible by 30 J/m^2 UVC to HT29, HT29/P14 and HT29/A11 cells (Figures 17 and 18). However, p53 could be shown to be inducible in HCT/116 in all four experiments conducted. This experiment shows that HT29/A11 and HT29/P14 are not capable of inducing their p53 protein expression levels in response to 30 J/m^2 UVC, which is a sufficient dose to lower cell clonogenic survival to 10% or less. This suggests that the inducible p53 response is absent in HT29 cells and their PDT-resistant variants, and that upregulation of p53 is not involved in cellular response to UVC in the HT29 clonogenic variants.

Figure 17: p53 and actin protein expression levels as detected by Western blot for HCT/116, HT29/Parental, and HT29/All cells 24 hours post treatment with 30 J/m^2 UVC. Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel. After Western blots were probed with anti-p53, they were stripped and re-probed with anti-actin to provide a loading control. This experiment was performed in duplicate.

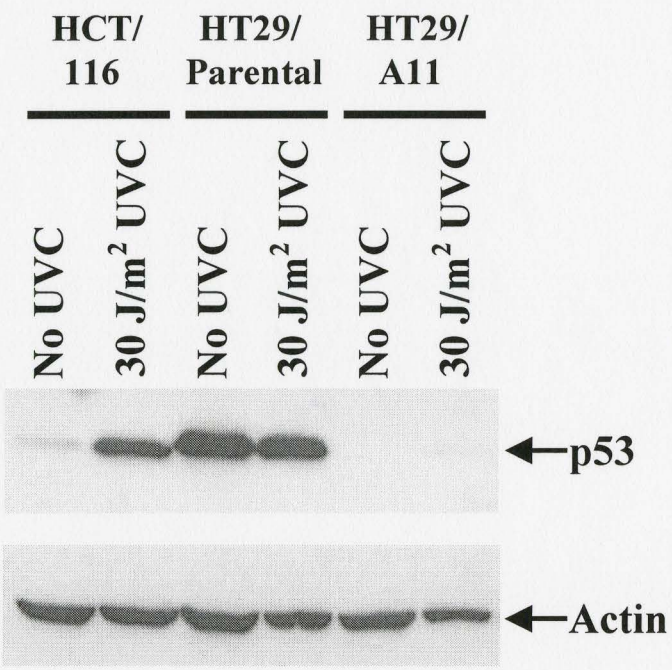
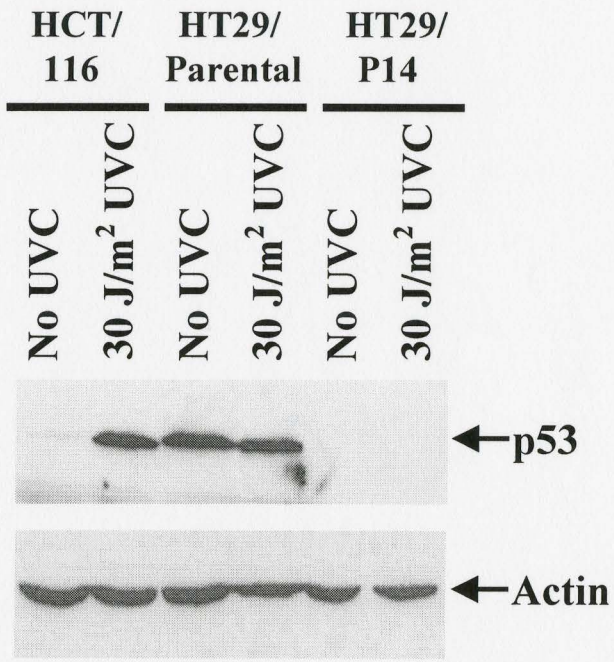

HCT/ 116
HT29/ Parental
HT29/ A11
No UVC
30 J/m^2 UVC
No UVC
30 J/m^2 UVC
No UVC
30 J/m^2 UVC
p53
Actin

Figure 18: p53 and actin protein expression levels as detected by Western blot for HCT/116, HT29/Parental, and HT29/P14 cells 24 hours post treatment with 30 J/m^2 UVC. Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel. After Western blots were probed with anti-p53, they were stripped and re-probed with anti-actin to provide a loading control. This experiment was performed in duplicate.


HCT/ 116
HT29/ Parental
HT29/ P14
No UVC
30 J/m2 UVC
No UVC
30 J/m2 UVC
No UVC
30 J/m2 UVC
p53
Actin

4.2 p21 Induction Assays Using Cisplatin

Since the high p53 protein expression levels in the HT29 control cell lines were correlated to resistance to cisplatin, it was proposed that perhaps p53 is needed in HT29 cell lines to induce p21, which causes cell-cycle arrest, giving cells time to repair the DNA damage. This hypothesis was tested by examining whether p21 could be differentially induced by cisplatin in HT29/Parental and HT29/P14 cells. HCT/116 cells were used in this experiment as well since it has a wild-type p53 and a known inducible p21 in response to cisplatin treatment. Cells were seeded and allowed to grow to semi-confluency, and treated with varying doses of cisplatin for 1 hour. Twenty-four hours post cisplatin treatment, the cells were turned into protein lysates which were ran out for western blots which were probed with anti-p21. p21 protein expression levels were compared to actin protein levels as a control obtained through stripping and re-probing each blot with anti-actin.

p21 levels could not be shown to be inducible in HT29/Parental or HT29/P14 by various cisplatin concentrations examined in two separate experiments (see Figures 19 and 20). However, 24 hours post 60, 80 and 160 μM cisplatin treatments for 1 hour, p21 protein levels were induced in HCT/116 cells. In another experiment using only 30 μM cisplatin treatment to cells, p21 levels were shown not to be induced even in HCT/116 cells (data not shown). Therefore the differences observed in cell sensitivity to cisplatin by HT29/Parental and HT29/P14 can not be attributed to a difference in cell cycle arrest induction by p21 as a result of varying mutant p53 levels. It appears likely that the mutation in p53 prevents it from being able to upregulate p21 levels.

Figure 19: p21 and actin protein expression levels as detected by Western blot for HCT/116, HT29/Parental, and HT29/P14 cells 24 hours post 60 μM cisplatin treatment for 1 hour. Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel. After Western blots were probed with anti-p21, they were stripped and re-probed with anti-actin to provide a loading control. Results from a single experiment.

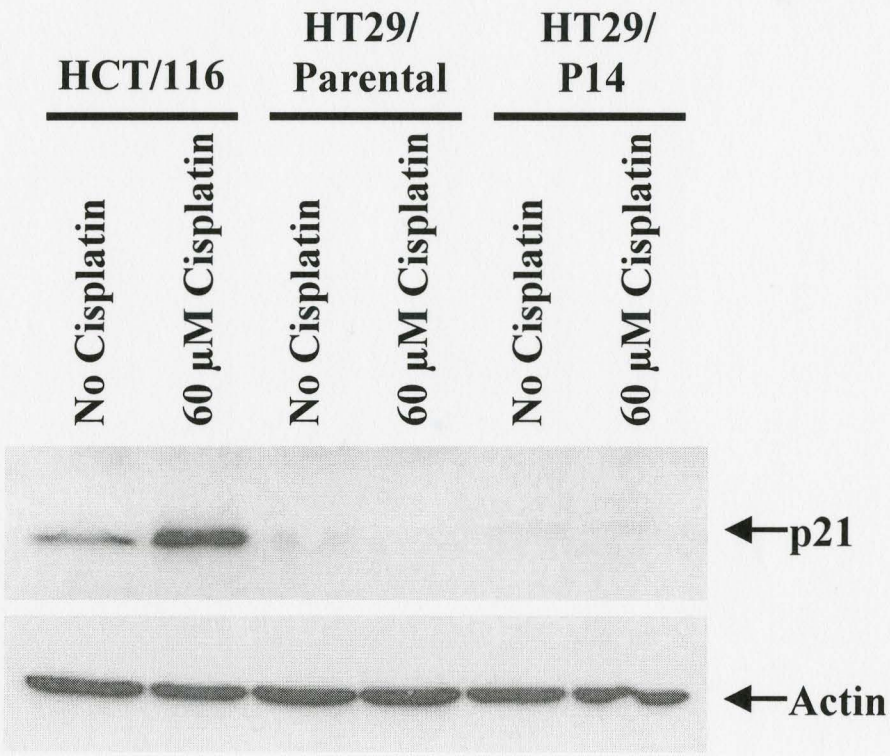
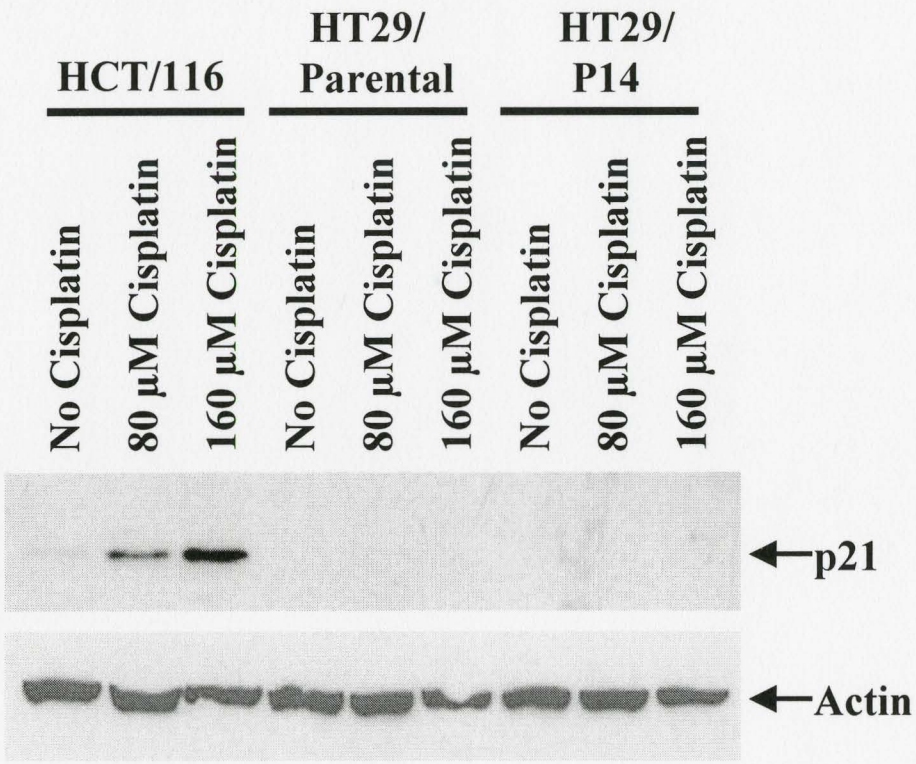

HCT/116
HT29/ Parental
HT29/ P14
No Cisplatin
60 µM Cisplatin
No Cisplatin
60 µM Cisplatin
No Cisplatin
60 µM Cisplatin
p21
Actin

Figure 20: p21 and actin protein expression levels as detected by Western blot for HCT/116, HT29/Parental, and HT29/P14 cells 24 hours post 80 and 160 μM cisplatin treatment for 1 hour. Equal amounts of complete cell lysates were run on 10% SDS-PAGE gel. After Western blots were probed with anti-p21, they were stripped and re-probed with anti-actin to provide a loading control. Results from a single experiment.

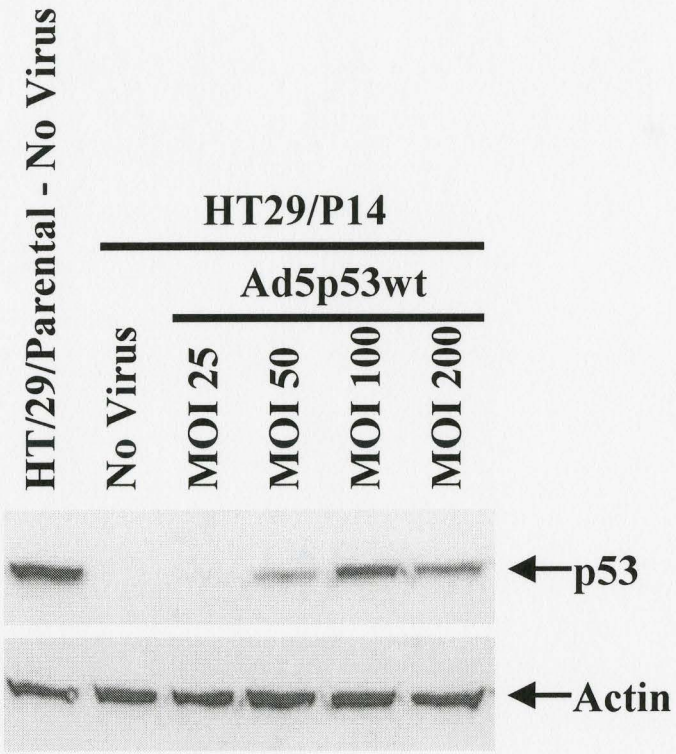

HCT/116
HT29/ Parental
HT29/ P14
No Cisplatin
80 µM Cisplatin
160 µM Cisplatin
No Cisplatin
80 µM Cisplatin
160 µM Cisplatin
No Cisplatin
80 µM Cisplatin
160 µM Cisplatin
p21
Actin

5. Colony Survival Assays With Cisplatin Using Cells Infected with Adenovirus Containing the Wild-Type p53 Gene

All three PDT-resistant cell lines were shown to under express mutant p53 and were more sensitive to cisplatin and UVC. In addition, high mutant p53 protein expression levels, were shown to correlate with cisplatin resistance and UVA sensitivity. An experiment was designed involving the expression of wild-type p53 in HT29/P14 and HT29/Parental through adenoviral transfer, in order to examine if p53 was indeed responsible for the differential sensitivity of the HT29 control and the PDT-resistant HT29 cell lines to cisplatin. p53 was re-expressed in the PDT-resistant cell lines through infection with Ad5p53wt, which contains the wild-type p53 gene under an HCMV promoter, and cellular sensitivity to cisplatin was examined using clonogenic survival assays for any changes. Control cells were or were not infected with AdCA17, which is an adenovirus that contains the β-galactosidase gene under the HCMV promoter.

Preliminary experiments were conducted with varying MOIs of Ad5p53wt virus using HT29/P14 to determine which MOI would result in a similar wild type p53 protein expression level in HT29/P14 as the mutant p53 protein expression level observed in HT29/Parental cells. With increasing MOI, the wild type p53 protein was increasingly expressed as determined by Western blot (see Figure 21). It was determined that Ad5p53wt used at an MOI of 100 would be appropriate for the clonogenic survival assays with Ad5p53wt infected cells, since the MOI of 100 provided the optimal expression level of p53.

Figure 21: Western blot of HT29/P14 infected with varying MOI's of Ad5p53wt containing the gene for wild type p53. HT29/Parental and HT29/P14 not infected with any virus were loaded as controls. The blot was stripped and re-probed with anti-actin to confirm equal protein loading. Results from a single experiment.


HT/29/Parental - No Virus
HT29/P14
Ad5p53wt
No Virus
MOI 25
MOI 50
MOI 100
MOI 200
p53
Actin

For the colony survival assays, HT29/Parental and HT29/P14 cells were infected with Ad5p53wt, or AdCA17 as a control virus, at an MOI of 100 (or mock-infected with no virus). Twenty-four hours post-infection, the cells were used in a clonogenic survival assays with cisplatin. For two of the three experiments conducted, any remaining cells not used for the clonogenic survival assays were turned into lysates and ran out for Western blotting to confirm p53 protein expression was increased in cells infected with Ad5p53wt. Typical results of the p53 protein expression levels observed in these experiments are shown in Figure 22. The p53 protein expression levels looked approximately the same in HT29/Parental cells mock infected with no virus, and infected with AdCA17 or Ad5p53wt. However, HT29/P14 cells infected with Ad5p53wt showed increased protein expression levels in comparison to mock infected or HT29/P14 cells infected with AdCA17, but not to the same level observed in the HT29/Parental cells for all three viral treatments.

Typical results from the colony survival assays with cisplatin using the infected HT29/Parental and HT29/P14 cells are shown in Figure 23 and the average D_{37} values for three experiments are listed in Table 10. Both HT29/Parental and HT29/P14 cells infected with AdCA17 or Ad5p53wt showed a slight increase in cisplatin sensitivity over uninfected cells. This suggested that the viral infection alone causes sensitization of HT29 cells to cisplatin. Both HT29/P14 and HT29/Parental cells infected with Ad5p53wt both showed an increase in sensitivity to cisplatin compared to their corresponding mock- or AdCA17-infected controls. For HT29/P14, the increased

sensitivity observed in Ad5p53wt-infected cells in response to cisplatin, was not significantly different from the AdCA17 infected HT29/P14 control used, but was significantly different from the HT29/P14 cells untreated with virus. There was no significant difference in cisplatin sensitivities observed in the HT29/Parental cells with any of the three viral treatments. The results from this experiment indicate that re-expressing wild-type p53 into HT29/P14 cells through adenoviral transfer, does not confer resistance to cisplatin.

Figure 22: Western blot of HT29/Parental and HT29/P14 infected with Ad5p53wt and AdCA17 both at MOI of 100. HT29/Parental and HT29/P14 not infected with any virus were loaded as controls. The blot was stripped and re-probed with anti-actin to confirm equal loading. Western blots were made from lysates from two separate experiments with the same results.

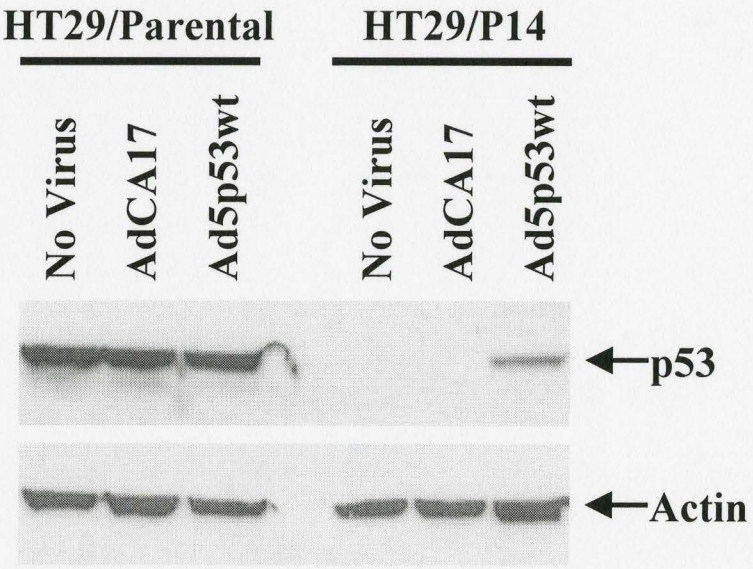
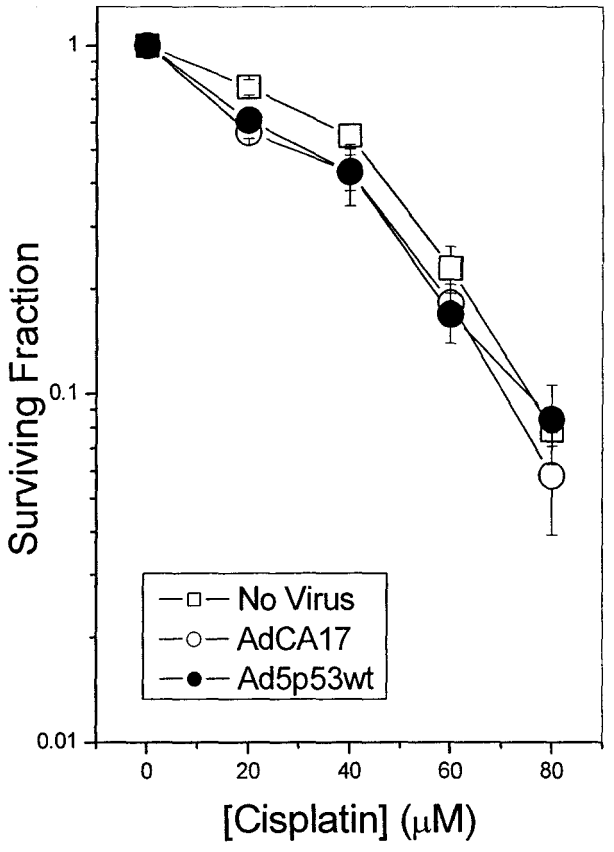

HT29/Parental
HT29/P14
No Virus
AdCA17
Ad5p53wt
No Virus
AdCA17
Ad5p53wt
p53
Actin

Figure 23: Colony survival assays for HT29/Parental (A) and HT29/P14 (B) cells that were infected with Ad5p53wt virus (□) containing the wild type p53 gene, or AdCA17 (○) as the control virus, or with no virus (●), and then treated with varying concentrations of cisplatin.

A. HT29/Parental



B. HT29/P14

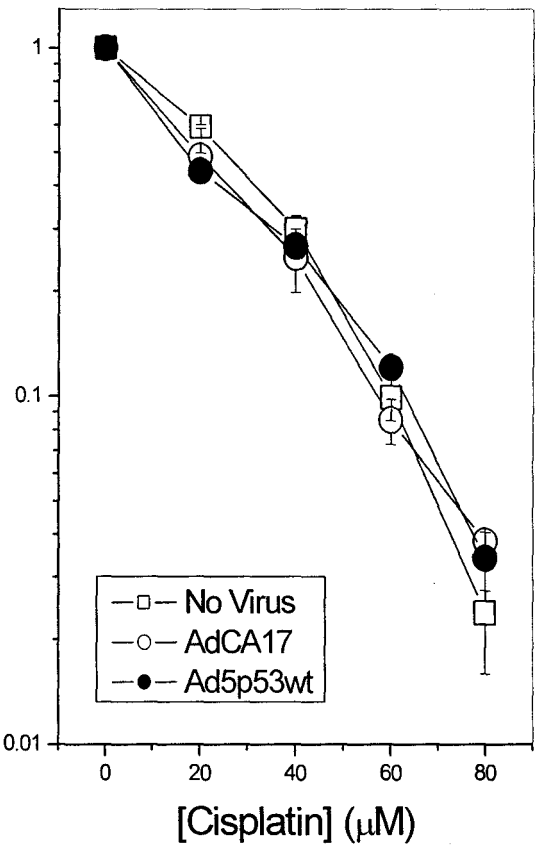


Table 10: Summary of the average $D_{37} \pm$ SE taken from the colony survival assays using cisplatin for HT29/Parental and HT29/P14 cells treated with AdCA17, Ad5p53wt, or no virus. HT29/Parental or HT29/P14 cells were infected with or without virus at MOI 100 for 90 minutes. Twenty-four hours post infection, cells were seeded for colony survival assay with cisplatin. The D_{37} was obtained in each experiment where each data point on the survival curve was the average ± SE of triplicate determinations. Each experiment was repeated three times and the average ± SE is listed in the following table. The D37 relative to HT29/P14 untreated with virus was calculated within each experiment and averaged for all three experiments.

Cell Line	Viral Treatment	Average Absolute D37	Average Relative D37
HT29/Parental	**No Virus**	38.48 ± 5.48	1.319 ± 0.108
	AdCA17	33.27 ± 5.29	1.133 ± 0.082
	Ad5p53wt	29.29 ± 6.89	0.987 ± 0.145
HT29/P14	**No Virus**	29.00 ± 2.46	1 ± 0
	AdCA17	25.18 ± 1.36	0.873 ± 0.025
	Ad5p53wt	21.64 ± 3.20	0.740 ± 0.063

6. Preliminary Apoptosis Assays

Since it was discovered that the pro-apoptotic factor BNip3 is over-expressed in all three PDT-resistant HT29 cell lines, it was of interest to examine if they undergo apoptosis more readily in response to cisplatin than the more cisplatin-resistant HT29/Parental cells. Cells were treated with varying concentrations of cisplatin for one hour and examined for apoptosis 24 or 72 hours later, using the TUNNEL assay as per the *In Situ* Cell Death Detection Kit, AP (Roche). With this assay, apoptotic cells stain purple upon exposure to BCIP/NBT, and therefore purple-stained cells were counted in four quadrants as a percentage of unstained cells under a light microscope and four counts were averaged for each cell line/treatment.

Figure 24 shows the results of apoptosis assays conducted twenty-four hours post 80 (A) and 160 μM (B) cisplatin treatment to cells for 60 minutes. After 80 μM cisplatin treatment to cells (Figure 24A), there is a slight but insignificant increase in the percentage of apoptotic cells observed for HT29/P14 cells. Apoptosis was not observed at all for HT29/Parental cells. However, no positive control was conducted for this experiment making it impossible to determine if the differences observed in these two cell lines are real. In addition, the percentage of apoptotic cells observed in HT29/P14 were extremely low (less than 0.5%) even for cells treated with cisplatin.

For an apoptosis assay conducted 24 hours post 160 μM cisplatin treatment to cells, the percentage of apoptotic cells was around 1% for both HT29/Parental as well as HT29/P14, and 0.05% for each of the corresponding untreated cells (Figure 24 B). The percentage of apoptotic cells observed in the treated cells was significantly greater than

non-treated cells for HT29/P14 ($P = 0.002$) but not for HT29/Parental cells. The increase in the percent apoptotic cells observed with cisplatin treatment was more pronounced with the increased dose of cisplatin used (160 as opposed to 80 μM). However, the positive control only showed approximately 10 % apoptotic cells. This indicates that the detection of apoptosis was not very efficient in this assay. In addition, HCT/116 did not show any detectable apoptosis even with 160 μM cisplatin treatment to cells.

Since some studies have shown that the percentage of apoptotic cells increased over time post cisplatin treatment, an apoptosis assay was performed seventy-two hours post 80 μM cisplatin treatment to cells (see Figure 25). There was an increase in the amount of apoptotic cells observed for untreated as well as treated HCT/116, HT29/P14, and HT29/Parental cell lines over the apoptosis assays performed twenty-four hours post cisplatin treatment. However, untreated HT29/Parental cells showed a higher percentage of apoptotic cells compared to cisplatin treated HT29/Parental cells, but cisplatin treated HT29/P14 cells showed more apoptosis compared to untreated cells. Since it is possible that untreated HT29 cells grow faster that cisplatin treated HT29 cells, an explanation for the increased percentage of apoptotic untreated HT29 cells over treated cells may lie in apoptosis being induced in untreated HT29 cells due to crowding after three days growth.

A repeat of this apoptosis assay was performed except that cells were grown directly on chamber slides and seeded at a much lower density to prevent cell crowding in the untreated control. However, the final two steps of the apoptosis assay were eliminated as per the proper manufacturers protocol, so that fluorescently labelled apoptotic cells could be visualized using fluorescence microscopy. This was done to

eliminate the AP converter step that transforms the fluorescent signal to a colourimetric one, in case this step was not working efficiently. Fluorescent staining could not be observed in the DNase treated positive control cells or for any of the other cell lines or treatments conducted.

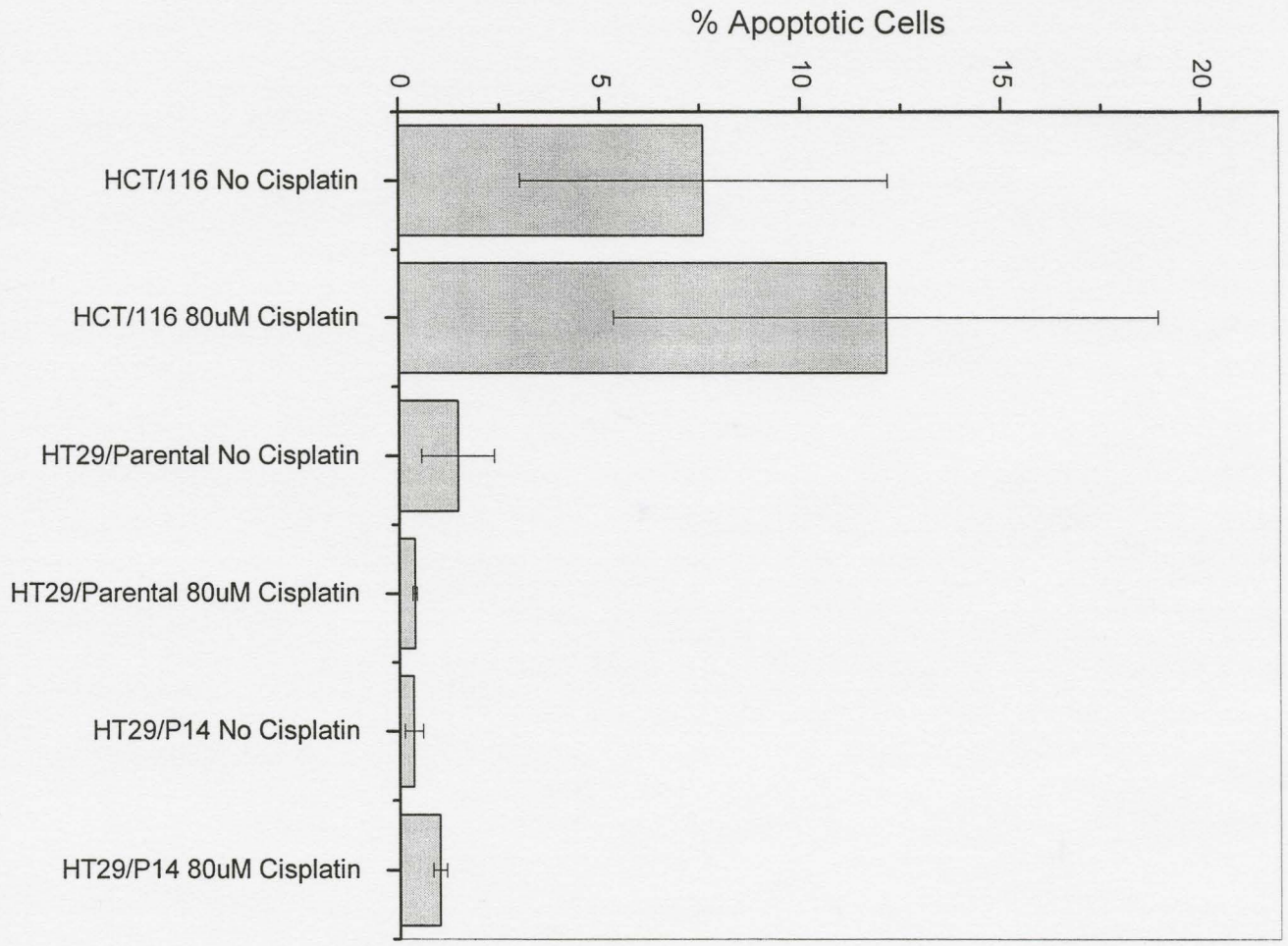
One last apoptosis assay was attempted where cells were examined for apoptotic activity twenty four hours post 0, 80, 160 and 320 μM cisplatin treatment for one hour. The fluorescence microscopy visualization of apoptotic cells again showed no apoptotic cells in any of the cell types or treatments including the DNase treated positive control showing that the assay failed.

Taken together, all of these results seem to indicate that the apoptosis assay was not working efficiently. More apoptosis assays need to be conducted until a proper positive control can be established such that any differences in apoptosis in HT29/Parental or HT29/P14 cells can properly be established.

Figure 24: Cells were seeded to confluency in 100 mm^2 petri dishes. Twenty-four hours post seeding, cells were treated with or without 80 μM (A), or 160 μM cisplatin (B) for 1 hour. These two apoptosis assays were conducted twenty-four hours post cisplatin treatment. For the negative control, cells were treated with label solution without terminal transferase enzyme. For the positive control, after permeabilization, cells were treated with DNase for 10 minutes before treatment with terminal transferase enzyme.

A
Percent Apoptotic Cells
0.0
0.1
0.2
0.3
0.4
0.5
No Cisplatin
NegativeControl
No Cisplatin
80 µM Cisplatin
HT29/Parental
No Cisplatin
80 µM Cisplatin
HT29/P14
B
Percent Apopototic Cells
0
2
4
6
8
10
12
HT29/P14 - No Cisplatin – Negative Control
HT29/Parental – No Cisplatin – Positive Control
HT29/Parental
No Cisplatin
160 µM Cisplatin
HT29/P14
No Cisplatin
160 µM Cisplatin
No Cisplatin
160 µM Cisplatin

Figure 25: Cells were seeded to confluency in 100 mm^2 petri dishes. Twenty-four hours post seeding, cells were treated with or without 80 μM cisplatin treatment for 1 hour. The apoptosis assay was conducted seventy-two hours post cisplatin treatment.


% Apoptotic Cells
0
5
10
15
20
HCT/116 No Cisplatin
HCT/116 80uM Cisplatin
HT29/Parental No Cisplatin
HT29/Parental 80uM Cisplatin
HT29/P14 No Cisplatin
HT29/P14 80uM Cisplatin

DISCUSSION

DISCUSSION

The HT29 PDT resistant variants were not cross-resistant to cisplatin or UVC. HT29/P14, HT29/A11 and HT29/N8 all showed a significant increase in cisplatin sensitivity of about 1.7 fold. This contrasts another study with a different photodynamic-therapy induced resistant cell line (RIF-8A) which did show cross-resistance to cisplatin (Moorehead *et al.*, 1994). Whatever the mechanism is that gives the HT29 PDT-resistance, it somehow makes them more sensitive to cisplatin. There are several ways in which cells can become resistant to cisplatin: increased DNA repair, increased production of thiol species, decreased drug uptake or increased drug efflux, or altered apoptotis (for review see Dempke *et al.*, 2000). If there are ways in which cells can gain resistance, there must also be ways in which cells can be sensitized to cisplatin perhaps through the loss of one of these mechanisms. Colon cancer cells have been shown to have an intrinsic resistance to cisplatin (Perez, 1998). Perhaps, the selection for PDT-resistance somehow makes HT29 cells lose this intrinsic resistance to cisplatin.

HT29/PcDNA showed a 2.35 fold significant increase in resistance to cisplatin, compared to the pooled HT29 controls (HT29a and HT29/Parental), while HT29/BNIP3 showed a similar sensitivity to the HT29 controls. This suggests that the insertion of the empty vector in HT29/PcDNA cells somehow induces HT29 cells to be more resistant to cisplatin. Interestingly, all vector-transfored cell lines showed higher levels of Hsp27. Perhaps, the increased resistance induced by the inserted vector in the HT29/BNIP3 cells,

is counteracted by the over expressed BNip3 protein, causing HT29/BNIP3 cells to show the same sensitivity to cisplatin as HT29 which has no inserted vector. These results suggest that BNip3 may be an important protein involved in HT29 cellular response to cisplatin.

HT29/A11 cells also showed a significant increase in UVC sensitivity of about 1.5 fold, whereas the UVC sensitivity of HT29/P14 and HT29/N8 was also less, but not significantly less from that of parental HT29 cells. The increased cisplatin sensitivity of these cells may carry over into cellular response to UVC since UVC and cisplatin both cause DNA damage repaired by the NER pathway, and may therefore trigger common cellular responses. Although HT29/BNIP3 and HT29/PcDNA were not significantly different from each other in cellular sensitivity to UVC, HT29/BNIP3 and its control (HT29/PcDNA) both showed significant increased sensitivity to UVC compared to HT29. One possibility is that the insertion of the vector in HT29 cells makes them slightly more sensitive to UVC, but BNip3 is not involved in cellular response to UVC. Another possibility is that the difference in the response of these cell lines to UVC is the result of clonal selection.

Out of the PDT-resistant HT29 variants, only HT29/P14 was significantly cross-resistant to UVA/B relative to HT29a by about 1.3 fold. In addition, the Hsp27 over expressing cell line, HT29/H13, and its control, HT29/Ne015, both showed significantly higher resistance to UVA/B. However it was difficult to determine the cellular sensitivity of the PDT-resistant cell lines in response to UVA/B compared to HT29/Parental, since in the single experiment where HT29/Parental cells were assayed for clonogenic survival

using UVA/B, they showed an increased resistance over HT29a by 1.6 fold, although this difference was not significant. Not enough experiments were conducted to determine whether HT29a responds to UVA/B in the same way that HT29/Parental does.

The HT29 PDT resistant variants were cross-resistant to UVA relative to HT29a. HT29/N8, and HT29/P14 both showed a significant increase in UVA resistance of about 1.6 and 1.4 fold compared to HT29a, respectively, whereas the HT29/A11 showed an increase of 1.2 fold, which was not significantly different from that of HT29a cells. In addition to oxidative damage, UVA has been shown like UVC to produce some CPD's (Douki *et al.*, 1999). Since HT29/A11 was also most sensitive to UVC and cisplatin, if it is deficient in DNA repair, this could offer an explanation as to why HT29/A11 is also more sensitive to UVA. However, again it was difficult to determine the cellular sensitivity of the PDT-resistant cell lines in response to UVA compared to HT29/Parental, since in the single experiment where HT29/Parental cells were assayed for clonogenic survival using UVA, they showed an increased resistance over HT29a by 1.3 fold, although this difference was not significant. However, not enough experiments were conducted to determine whether the response of HT29a to UVA was significantly different to that of HT29/Parental.

HT29/A11 appears to by significantly more cross-resistant to Photofrin-mediated PDT compared to the HT29/Parental while HT29/P14 showed only a slight insignificant increase in resistance and HT29/N8 was significantly more sensitive to Photofrin-mediated PDT. To complicate matters, there appears to be clonogenic variability among HT29 cell lines in response to Photofrin-mediated PDT, since HT29a shows a

significantly higher resistance to Photofrin-PDT than the HT29/Parental cell line, which was used to derive the PDT-resistant cell lines. Photofrin drug uptake levels were not measured in the three PDT-resistant cell lines. Therefore, the different responses observed in the three PDT-resistant cell lines and the two control HT29 cell lines to Photofrin-mediated PDT may have been the result of different levels of Photofrin drug uptake. In addition, clonogenic survival using Photofrin-mediated PDT was measured in this study as a function of light exposure for a given Photofrin concentration. However, for the original Photofrin-mediated PDT clonogenic survival assays used to establish and test HT29/P14, were performed as a function of Photofrin concentration for a given light exposure (Singh *et al.*, 2001). Perhaps our protocol for PDT is not as sensitive in detecting PDT resistance in HT29/P14.

Differences in DNA repair capabilities have been implicated as one of the possible means of cellular resistance to cisplatin (as reviewed by Kartalou and Essigmann, 2001). To examine if there were differences in DNA repair between HT29 and the most cisplatin sensitive PDT resistant cell line HT29/A11, HCR assays were performed with Ad5HCMVSP1LacZ virus damaged with UVC. HCR assays performed with varying MOIs of irradiated virus to untreated cells, showed that at the high MOI (80), HT29/A11 showed a reduced HCR capability. At higher MOIs, more damaged DNA gets inside the cells. Therefore a reduced HCR in HT29/A11 compared to HT29a at high MOIs suggests that HT29/A11 is repairing less of the damaged DNA introduced into the cell. This suggests that HT29/A11 is not as efficient at DNA repair as is HT29a. Perhaps it is the reduced DNA repair ability of HT29/A11 that causes it to be more

sensitive to UVC or cisplatin compared to HT29a. However, there were concerns that HT29/A11 cells were being infected more efficiently than HT29a under the same experimental conditions, and therefore MOIs that resulted in similar viral expression as measured through the rate of β-galactosidase reporter expression were examined. At MOIs that gave roughly equivalent rates of β-galactosidase reporter expression, there appeared to be no difference in HCR capabilities of these two cell lines.

UV enhanced reactivation (UVER) of the β-galactosidase reporter gene was not observed in HT29 cells. However, in HT29/A11, UVER was seen at the higher UVC dose examined (20 J/m^2 to cells). This actually implies that UV radiation induces DNA repair more in HT29/A11 than in HT29 cells, which contradicts the hypothesis that HT29/A11 might be more sensitive to UVC or cisplatin due to a deficient DNA repair system.

It has been previously proposed that UVC treatment to cells results in signals that may enhance the transcription and expression of the reporter gene through increased activation of the CMV promoter (Francis and Rainbow, 2000). It has been shown that lower UV exposures to TCR-deficient fibroblast strains, resulted in increased β-galactosidase activity from a CMV promoter, compared to higher UV exposures needed to give the same results in TCR-proficient fibroblast cell strains (Francis and Rainbow, 2000). Since TCR-proficient cell strains repair DNA damage in active genes, these results suggest that it is actually the unrepaired DNA damage in active genes that cause increased reporter activity (Francis and Rainbow, 2000). Pre-irradiating HT29a and

HT29/A11 with UVC also resulted in higher β-galactosidase expression of untreated reporter gene with increasing UVC dose to cells. However, no significant difference was observed in β-galactosidase expression levels between HT29 and HT29/A11 indicating that there was no difference in TCR capabilities between these two cell lines.

Taken together, the HCR results show no clear evidence for a difference in the DNA repair capabilities between these two cell lines. Therefore, based on the HCR results, the increased UVC or cisplatin sensitivity of HT29/A11 cannot be explained by lower repair rates

The basis of PDT resistance appears to be multifactorial in that more than one gene is probably involved: Hsp27, p53, BNip3, Bcl2 and Bax. All three PDT resistant variants showed increased levels of the Hsp27 (Wang *et al.*, 2002) and the anti-apoptotic Bcl-2 protein, decreased pro-apoptotic Bax protein expression levels, decreased levels of mutated p53, but increased levels of pro-apoptotic BNip3 (Shen *et al.* 2000). The combination of protein expression levels that provides these cells PDT resistance may commonly provide cells UVA resistance but make them more sensitive to cisplatin and UVC. The common resistance to UVA may arise since UVA causes oxidative damage to cells that may be similar to that caused by PDT. It is interesting that no correlation was observed between the Hsp27 or p53 protein expression levels examined and UVC sensitivity. UVC resistance in cells may be mediated through means other than through these protein expression levels. It could not be elucidated in this study whether differences in UVC sensitivity were the result of differences in DNA repair capabilities.

Hsp27 protein expression levels are stably increased in all three PDT-resistant HT29 cell lines. It is possible that Hsp27 is uniquely involved in making HT29 cells more resistant to PDT since Hsp27 levels did not correlate with sensitivity to the other agents tested (UVC, UVA and cisplatin). In addition to the chaperone activity, and actin filament stabilization which Hsp27 exhibits, another possible explanation for the selection of the Hsp27-overexpressing PDT-resistant HT29 cell lines lies in the recent findings that Hsp27 is involved in apoptosis, and acts as an anti-apopotic molecule (for review see Concannon *et al.*, 2003). Hsp27 has been shown to negatively regulate cell death by interacting with cytochrome-*c* released from the mitochondria and preventing cytochrome-*c*-mediated interaction of Apaf-1 with procaspase-9 (Bruey *et al.*, 2000). Another study implies that Hsp27 actually represses caspase-3 activation (Pandy *et al.*, 2000). Photosensitizers that localize in the mitochondria are rapid apoptosis inducers (as reviewed by Moor, 2000). Although the mitochondria may not be the primary target of action for Nile Blue A or aluminum phthalocyanine tetrasulfonate, experiments also have not completely ruled out the mitochondria as a site of action for these photosensitizers. Perhaps the increased Hsp27 in all three HT29 PDT-resistant cell lines inhibits apoptosis in these cell lines in response to PDT, providing them PDT-resistance.

Several studies show that Hsp27 is involved in cellular resistance to cisplatin (Huot *et al.*, 1991; Langdon, *et al.*, 1995; Oesterreich *et al.*, 1993; and Yamamoto *et al.*, 2001). Increased Hsp27 protein expression levels has been implicated in mediating resistance to cisplatin in CHO cells (Huot *et al.*, 1991), breast cancer tumours (Oesterreich *et al.*, 1993), and ovarian carcinomas (Arts *et al.*, 1999; and Yamamoto *et*

al., 2001). A PDT resistant HT29 cell line made to over express Hsp27 alone showed a similar sensitivity to that of parental HT29 cells following cisplatin. However, there was not a significant difference between Hsp27 protein expression levels observed by western blot in the Hsp27 over expressing cell line and its control. Even so, we can still conclude that Hsp27 over expression alone is not responsible for the cisplatin sensitivity of the HT29 PDT resistant variants since there was no correlation of Hsp27 protein expression levels to $1/D_{37}$ for the cisplatin colony survival assays. The cisplatin resistance observed in this study in the PDT-resistant HT29 cell lines, must therefore be the result of some other mechanism.

UVA creates reactive oxygen species in cells (Kielbassa et al., 1997), and Hsp27 has been shown to protect cells against reactive oxygen species (Huot *et al.*, 1995; and Spitz *et al.*, 1987). However, since the Hsp27 protein expression levels did not correlate with HT29 cell sensitivity to UVA, it indicates that Hsp27 may not be involved in the increased cellular resistance observed by the PDT-resistant HT29 cell lines in response to UVA. Like Hsp27, BNip3 protein expression levels also did not correlate with UVA HT29 cell sensitivities. In contrast, p53 protein expression levels did correlate with sensitivity of cells to UVA suggesting that p53 (even though mutated in HT29 cells) could be important in the cellular response of HT29 to UVA.

A correlation of cellular sensitivity in response to a mixture of UVA and UVB (UVA/B) to the different measured protein expression levels was not determined. It would be interesting to make the correlation to see whether p53 is involved in cellular sensitivity to UVA/B. HT29/P14 did show a 1.34-fold significant increase in resistance

to UVA/B over HT29. However, in the only colony survival assay that included the parental HT29 cell line, HT29/Parental was observed to be almost 1.55-fold more resistant to UVA/B than HT29. This shows that the PDT-resistant HT29 cell lines might actually be more sensitive to UVA/B than their parental HT29 cell line. However, since the parental HT29 cell line was only assayed once for sensitivity to UVA/B, it makes it difficult to determine whether the PDT-resistant cell lines are more resistant or more sensitive to UVA/B.

If the PDT-resistant cell lines are indeed more sensitive to UVA/B and UVC than the parental HT29 cell line, but are more resistant to UVA, then this suggests that UVA and UVB/UVC might kill cells through different mechanisms. A study by Wang and colleagues (1998) showed that apoptosis occurred much earlier in MCF-7 cells exposed to UVA than in cell exposed to UVB (Wang *et al.*, 1998). In addition, increasing exposures of UVA to cells resulted in decreased Bcl-2 protein expression levels and unchanged p53 protein expression levels. However, for UVB exposure to cells, Bcl-2 protein expression levels remained unchanged, whereas p53 protein expression levels increased (Wang et al., 1998). This study suggests that UVA and UVB affect cells differently and these effects are mediated through Bcl-2 and p53. Since the PDT-resistant cell lines express different levels of these two proteins compared to HT29/Parental cells, it is possible that they could be differentially sensitized to UVA compared to UVA/B and UVC. However, since MCF-7 cells express wild-type p53, and HT29 cells express mutated p53, it is difficult to determine how UVA and UVB may differentially affect HT29 cells unless the mutation in p53 does not affect this function. In addition, the UVA

source used in this project actually still contained a small percentage of UVB (5.62%). Also, the mixture of UVA/B probably results in a combination of effects on cells by both UVA and UVB-mediated mechanisms.

p53 has been implicated numerous times to be involved in the cellular response to Photofrin-mediated PDT (Fisher *et al.*, 1998; Tong *et al.*, 2000; and as reviewed by Dougherty *et al.*, 1998). In one study, Li-Fraumeni Syndrome (LFS) cells which have a mutated p53 have been shown to be more resistant to Photofrin-mediated PDT than normal human fibroblasts (Tong *et al.*, 2000). In addition, adenovirus transfer of the wild-type p53 gene in LFS cells resulted in increased sensitivity to PDT (Tong *et al.*, 2000). In addition to killing cells through necrosis, apoptosis has been thought to be an important means of cell killing by PDT (Gupta *et al.*, 2003; as reviewed by Dougherty *et al.*, 1998). Since all three PDT-resistant HT29 cell lines were shown by western blot to underexpress p53, one hypothesis is that PDT kills HT29 cells through p53-mediated apoptosis. However, HT29 cells have a mutated p53 and p53 mutations have often been implied to disrupt apoptosis (Zhang *et al.*, 1999; and Hajri *et al.*, 2002). In addition, apoptosis has not always been shown to correlate with cellular sensitivity to Photofrin-mediated PDT (Tong *et al.*, 2000). And, some studies implicate that p53 expression does not directly modulate human tumour cell sensitivity to Photofrin-mediated PDT (Fisher *et al.*, 1999).

Human cancers almost always show aneuploidy, and the p53 gene is the most common genetic change found in human cancers (Hollstein *et al.*, 1991). In addition, most cell lines with mutant p53 genes, over express their mutant p53 protein

constitutively (Rodrigues *et al.*, 1990). In human colorectal cancer, it has been found that over expression of p53 is synonymous with mutation to the p53 gene perhaps because the mutation results in a more stable form of the p53 protein (Rodrigues *et al.*, 1990). Constitutive p53 protein overexpression was observed in both sources of HT29 cells examined by Western blot. In addition, wild type p53 protein accumulates in cells upon UV radiation (as reviewed in Decraene *et al.*, 2001), and p53 protein expression could not be shown to be inducible by exposing any of the three HT29 clonogenic variants examined to 30 J/m^2 UVC. This implies that the normal p53 accumulation upon UV exposure has been disrupted either as a result of the mutation in the p53 gene or through the diruption of some other pathway upstream of the p53 gene.

Although it has often been implied that mutated p53 cannot initiate apoptosis (Zhang *et al.*, 1999; and Hajri *et al.*, 2002), this may not be true for all types or all p53 mutations. HT29 specifically has a point mutation in p53 at codon 273 ($Arg^{273}His$) (Rodrigues *et al.*, 1990). The type of mutated p53 in HT29 can still bind and activate transcription of a reporter construct with p53 recognition sequences (Park *et al.*, 1994). Also, El-Hizawi and colleagues (2002) have shown that forms of mutant p53, especially the $Arg^{273}His$ mutated p53, interact with topoisomerase I, resulting in gene amplification and a gain-of-function oncogenic effect, making a cancer cell more likely to develop resistance to chemotherapeutic drugs (El-Hizawi *et al.*, 2002). The interaction between p53 and topoisomerase I is thought to lead to an even more increased genomic instability in these cells (El-Hizawi *et al.*, 2002).

Perhaps due to the instability of the genome in the HT29 tumour cells, it is possible that a flask of HT29 cells may actually consists of a population of tumour cells that express various levels of the mutant p53, the majority of the cells over expressing p53. During the selection process used to create the PDT resistant HT29 cell lines, PDT may have killed the PDT sensitive, highly expressing p53 cells from the cell population, leaving the p53 under expressing cells behind because they are more resistant to PDT. The lower p53 levels in the PDT resistant cell lines left behind may further increase genomic instability, accelerating acquisition of further mutations for resistance (Giannakakou *et al.*, 2000). In Li-Fraumeni syndrome, individuals inherit a germ-line mutation in one allele of p53 and eventually lose the remaining wild-type p53 gene probably due to genomic instablility (Malkin, 1994). It would be interesting to see if perhaps the p53 gene is deleted in all three PDT-resistant HT29 cell lines. Interestingly, upon examining the cDNA microarray conducted with HT29 and HT29/P14 (Wang *et al.*, 2002), it was observed that hybridization did not occur at the p53 gene for either cell line examined. It might be possible that the mutation in the p53 gene in HT29 makes is unable for the cellular p53 cDNA to hybridize with the p53 cDNA on the microarray, thus making a hybridization signal undetectable.

Several studies indicate that inactivation of p53 leads to enhanced sensitivity to multiple chemotherapeutic agents including cisplatin (Hawkins *et al.*, 1996; and Pestell *et al.*, 1999). However, other results suggest that overexpression of p53 leads to increased sensitivity to cisplatin (Fujiwara *et al.*, 1994; and Blagosklonny *et al.*, 1998). To examine whether the differences in cisplatin sensitivity between the PDT-resistant and parental

HT29 cell line were due to protein expression differences of p53, cells were infected with adenovirus containing a wild-type p53 gene. It was hypothesized that overexpressing wild type p53 in the PDT-resistant HT29 cell lines by infection with Ad5p53wt, might increase their cisplatin resistance since all three PDT-resistant cell lines were shown to be more sensitive to cisplatin and all showed reduced p53 protein expression levels. However, in contradiction to this hypothesis, the adenoviral introduction of wild-type p53 in HT29/P14 and HT29/Parental cells, actually made these cells slightly more sensitive to cisplatin. In agreement with these results, several studies have shown that the introduction of wild type p53 into cells by adenovirus, sensitizes them to cisplatin by accelerating cell death through apoptosis (Fujiwara *et al.*, 1994; and Blagosklonny *et al.*, 1998). However, the sensitization of HT29/P14 and HT29/Parental cells to cisplatin through the introduction of wild-type p53 was not significant compared to the AdCA17 infected control cells. Only for HT29/P14 was there a significant increase in sensitivity in both Ad5p53wt as well as AdCA17 infected cells over uninfected cells. The increased sensitivity of AdCA17 infected HT29a cells was not significant compared to uninfected HT29a cells. This experiment however, implies that merely infecting with adenovirus alone, sensitizes cells to cisplatin. The lack of sensitization upon introduction of wild-type p53 protein into HT29/Parental and HT29/P14 cells could be due to not enough wild-type p53 being introduced into the cells upon infection.

The PDT-resistant cell lines were actually more sensitive to cisplatin than the parental as well as the additional HT29 cell line examined by colony survival assays. In addition, all PDT-resistant cell lines showed greatly reduced p53 protein expression

levels. Perhaps upon cisplatin treatment, p53 (even the mutated p53 in HT29 cells) might be needed to induce p21 to arrest cells in G1, giving the cells time to repair the DNA damage. In support of this hypothesis, HCT/116 cells with a disrupted p21 gene have been shown to exhibit enhanced sensitivity to cisplatin over HCT/116 cells containing wild-type p21 (Fan *et al.*, 1997). Thus it was hypothesized that the PDT-resistant cell lines may be more sensitive to cisplatin as a result of their lower levels of p53 protein expression resulting in a lack of these cells' ability to induce p21. However, examination of p21 levels in HT29 and the HT29 PDT-resistant cell lines by Western blot showed that p21 levels are not detectable in untreated cells, or inducible in cells treated with cisplatin concentrations of up to 160 μM in both parental HT29 as well as the PDT-resistant HT29 cell lines. Therefore the differences observed in cell sensitivity to cisplatin by HT29/Parental and HT29/P14 cannot be attributed to a difference in cell cycle arrest induction by p21 as a result of varying mutant p53 levels. The fact that mutant p53 protein expression levels could not be shown to be inducible by UVC in HT29/Parental, or HT29/A11 and HT29/P14, provides an explanation as to why p21 levels are not inducible by cisplatin, since the induction of p21 is dependent on a functional p53.

p53 protein expression levels correlated negatively ($R = -0.82$, $P < 0.0001$) and BNip3 protein expression levels correlated positively ($R = 0.78$, $P < 0.0001$) with cisplatin sensitivity. The best protein expression level to cellular sensitivity to a damaging agent correlation was obtained from a plot of BNip3/p53 versus cisplatin sensitivity ($R = 0.89$, $P < 0.0001$) (data not shown). However, the correlation of BNip3/p53 to survival after UVA was actually worse ($R = -0.57$, $P = 0.002$) (data not

shown) than for the individual p53 plot. This suggests that the relative expression of BNip3 compared to p53 is an important determinant in cisplatin sensitivity in HT29 cells. The strong correlation of cellular cisplatin sensitivity to the ratio of BNip3 to p53 protein levels suggest that it is actually the relative expression of BNip3 compared to p53 that is an important determinant in cisplatin sensitivity in HT29 cells. It has been reported previously that apoptosis induced by BNip3 is significantly inhibited by both wild type and mutated p53 (Tsonuda *et al.*, 1999). If mutant p53 abrogates BNip3-mediated apoptosis in HT29 cells, this might explain why the ratio of BNip3 to p53 is an important determinant of cellular sensitivity to cisplatin. It appears likely that alterations in the expression of several different genes, including a reduced expression of the mutant HT29 p53 protein and an increased expression of BNip3, contribute to the increased cisplatin sensitivity of the HT29 PDT-resistant variants. On this basis, the significantly higher resistance of HT29/PcDNA cells to cisplatin compared to HT29/BNIP3 and HT29 cells may be explained, in part at least, by its low BNip3/p53 ratio of 0.0019 ± 0.0012 which was lower than that in HT29 (0.0045 ± 0.0012, P = 0.03) and HT29/BNIP3 cells (0.0261 ± 0.0219). This suggests that cisplatin kills HT29 cells through BNip3 mediated apoptosis while PDT might kill HT29 cells through a different pathway.

Apoptosis induced by BNip3 has been shown to be significantly inhibited by both wild type and mutated p53 in mouse cerebellum cells (Tsonuda *et al.*, 1999). If p53 abrogates BNip3-mediated apoptosis in HT29 cells, one would expect that the experiment where wild-type p53 was introduced into the BNip3 overexpressing PDT-resistant HT29/P14 cells through infection with Ad5p53wt virus, should have resulted in an

increased cellular resistance to cisplatin, as a result of a decrease in apoptosis in HT29/P14 cells. However, there was no difference in cellular reponse to cisplatin between Ad5p53wt infected and AdCA17 infected control cells. One explanation might be that perhaps not enough wild type p53 was introduced into the HT29/P14 cells to abrogate BNip3. Another explanation might be that the percentage of cells that die in response to cisplatin as a result of apoptosis is so small compared to the total amount of cell death, that any change in the apoptotic percentage is not detectable as a change in clonogenic survival.

BNip3/p53 protein expression levels correlated very well with cisplatin sensitivity. It was hypothesized that perhaps the differential sensitivity of the PDT-resistant and parental HT29 cell lines to cisplatin is the result of reduced apoptosis in the parental HT29 cell line compared to the PDT-resistant cell lines. If this is true, a possible explanation might be that cisplatin kills cells through BNip3-mediated apoptosis. BNip3 is a pro-apototic factor and is shown to be overexpressed in all three PDT-resistant HT29 cell lines, which could explain their increased sensitivity to cisplatin over the parental HT29 cell line and the other HT29 cell line examined in this study. Perhaps the cisplatin resistance in the HT29 control cell lines (HT29a and HT29/Parental), results from their lower pro-apoptotic BNip3 protein expression levels, compared to the PDT-resistant HT29 cell lines. Preliminary apoptosis assays were performed using cisplatin to investigate this hypothesis but unfortunately the results were inconclusive. Cisplatin treatment to cells resulted in a slight but insignificant increase in apoptotic activity twenty-four hours post 80 μM cisplatin treatment only in HT29/P14 cells. For an

apoptosis assay conducted 24 hours post 160 μM cisplatin treatment to cells however, the percentage of apoptotic cells was significantly greater than non-treated cells for HT29/P14 ($P = 0.002$) but not for HT29/Parental cells. However, the apoptosis assay was not very efficient due to the low detection of apoptotic cells in the assay as well as in the DNase treated positive control cells in the assay where 160 μM cisplatin was used.

BNip3 has been shown to induce a novel form of death resembling necrosis, which occurs independent of caspases and the Apaf-1/cytochrome *c* mitochondrial pathway typical of apoptosis (Vande Velde *et al.*, 2000). BNip3 has been shown to heterodimerize with Bcl-2 and Bcl-XL and induce cell death independent of the BH3 domain thought to be responsible for conferring pro-apoptotic activity in other BH3 containing proteins (Ray *et al.*, 2000). Several lines of evidence have pointed to BNip3 inducing cell death by causing the loss of membrane integrity (Vande Velde *et al.*, 2000). It is possible that cisplatin kills cells through BNip3 mediated necrosis-like death. Perhaps then, apoptosis might not be detectable at high levels upon cisplatin treatment of the PDT-resistant HT29 cells. As a result of the BNip3-overexpressioin in the PDT-resistant cell lines, they could be more sensitive to cisplatin than the control HT29 cell lines.

This study suggests that in order to determine whether a specific protein is involved in a cellular response to an agent, a variety of clonal variants of the same cell type must be tested in order to establish a correlation between protein expression levels and sensitivity. Clonal variability was observed in the HT29 control cell lines used. Also, the results observed with the HT29 cell lines made to overexpress BNip3 or Hsp27

alone compared to their empty vector controls and the unaltered cell lines, did not always clearly show whether each protein was involved in cellular sensitivity. Therefore, simply making an inference from one cell line with one altered protein expression will not always provide an accurate indication of its involvement. Without correlations it is difficult to determine if a protein is indeed involved in a cellular response.

This study suggests also that cells do not attain resistance to a damaging agent through just one mechanism. It was a combination of multiple changes in protein expression levels that gave the three different established HT29 cell lines their resistance to PDT. Similarly, Violette and colleagues (2002) found that a combination of multiple proteins such as Bcl-2, Bcl-XL, Bax and p53 were correlated to long-term resistance to 5-fluorouracil in colon carcinoma cells (Violette *et al.*, 2002). It is interesting that all three PDT-resistant cell lines although generated through repeated PDT exposure with three different agents, all exhibit similar protein expression profiles for the Hsp27, BNip3, Bcl-2, Bax, and p53 genes. It is therefore possible that resistance to PDT arises through the same mechanism regardless of the type of photosensitizer used for selection, and regardless of the intracellular localization site of the photosenstizer.

SUMMARY and FUTURE DIRECTIONS

SUMMARY

1. The HT29 PDT resistant variants were not cross resistant to cisplatin or UVC. HT29/P14, HT29/A11 and HT29/N8 all showed a significant increase in cisplatin sensitivity of about 1.7 fold. HT29/A11 cells also showed a significant increase in UVC sensitivity of about 1.5 fold, whereas the UVC sensitivity of HT29/P14 and HT29/N8 was greater but was not significantly greater than that of parental HT29 cells.

2. HT29/P14 was significantly cross-resistant to UVA/B relative to HT29a by about 1.3 fold. In addition, the Hsp27 over expressing cell line, HT29/H13, and its control, HT29/Ne015, were also both significantly more resistant to UVA/B. In a single experiment where HT29/Parental cells were assayed for clonogenic survival using UVA/B, they showed an increased resistance over HT29a by 1.6 fold, although this difference was not significant. Not enough experiments were conducted to determine whether HT29a responds to UVA/B in the same way that HT29/Parental does.

3. The HT29 PDT resistant variants were cross resistant to UVA relative to HT29. HT29/N8, and HT29/P14 both showed a significant increase in UVA resistance of about 1.6 and 1.4 fold, respectively whereas the HT29/A11 showed an increase of 1.2 fold, which was not significantly different from that of HT29 cells. However, not enough experiments were conducted to determine whether our HT29a responds to UVA in the same way that the parental HT29 does.

4. HT29/P14 showed a slight but insignificant increase in resistance to Photofrin-mediated PDT compared to HT29/Parental, while HT29/A11 was significantly more resistant than HT29/Parental. However, HT29/N8 was significanlty more sensitive to Photofrin-mediated PDT than HT29/Parental. To complicate matters, there appears to be clonogenic variability among HT29 cell lines since HT29a shows a significantly higher (1.7 fold) resistance to Photofrin-PDT than HT29/Parental.

5. We were unable to detect a difference in the DNA repair capabilities of UVC induced DNA damage between HT29 and HT29/A11 (the most sensitive cell line to UVC). However, due to differences in cellular morphology between HT29 and its derived PDT resistant cell lines, it is difficult to compare DNA repair in these cell lines using HCR assays.

6. A PDT resistant HT29 cell line over expressing Hsp27 alone showed a similar sensitivity to that of parental HT29 cells following cisplatin. However, there was not a significant difference between Hsp27 protein expression levels between the over expressing cell line and its control. Even so, we can still conclude that Hsp27 over expression alone is not responsible for the cisplatin sensitivity of the HT29 PDT resistant variants since there was no correlation of Hsp27 protein expression levels to $1/D_{37}$ for the cisplatin colony survival assays. Hsp27 protein expression levels did not correlate with UVC, cisplatin or UVA sensitivity showing that Hsp27 may be uniquely involved in making cells more resistant to PDT.

7. p53 but not BNip3 protein levels correlated with sensitivity of cells to UVA

8. There was a strong correlation of cellular cisplatin sensitivity to the ratio of BNip3 to p53 protein levels.

9. No correlation was observed between p53 or Hsp27 protein expression levels and UVC sensitivity. UVC resistance in cells may be mediated through other means than these protein levels. It could not be elucidated in this study whether differences in UVC sensitivity were the result of differences in DNA repair capabilities.

10. From results of p53 induction assay with UVC, and p21 induction assays with cisplatin, it was concluded that the increased sensitivity of the PDT-resistant cell lines in response to cisplatin, compared to parental HT29 cells, is not a result of a decreased ability to induce cell cycle arrest.

11. Introducing wild-type p53 into HT29/Parental and HT29/P14 cells by Ad5p53wt, did not significantly alter sensitivity of these cell lines to cisplatin compared to cells infected with a control virus (AdCA17).

12. Due to technical difficulties, preliminary apoptosis assays could not determine if there was a difference in the amount of apoptosis observed between HT29/P14 and HT19/Parental cell lines in response to cisplatin.

FUTURE DIRECTIONS

The results of this study have led to the hypothesis that the PDT-resistant HT29 cell lines are more sensitive to cisplatin than parental HT29 cells, due to an increased apoptotic response as a result of their increased BNip3 protein expression levels. Results from preliminary apoptosis assays performed on HT29/P14 and HT29/Parental cells after cisplatin treatement were inconclusive. It would be very valuable to perform more apoptosis assays with the proper conditions and controls for PDT-resistant HT29 cell lines as well as the control HT29 cells treated with cisplatin.

HT29a and HT29/Parental showed a significant difference in sensitivy in response to Photofrin-mediated PDT in this study. It would be of interest to examine whether these two cell lines also show clonal variability in response to UVA and UVA/B. If different sources of the same cell line respond differently to the same agent, then this should be born in mind in the interpretation of results from clonogenic survival assays using a single parental cell line.

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