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Experimental and Theoretical Improvements in the Investigation of Sonodynamic Cancer Therapy

by

Frederick Paul Ragan

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

in

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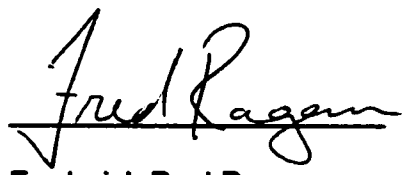

Frederick Paul Ragan
11406-66 Street
Edmonton, Alberta
T5B 1H8

17 April 2000

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Experimental and Theoretical Improvements in the Investigation of Sonodynamic Cancer Therapy* by Frederick Paul Ragan in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

14/04/2000
Gerald George Miller

Leonard Irving Wiebe

James William Lown

Stephen McQuarrie

Abstract

Sonodynamic therapy is an experimental tumour treatment which uses a combination of ultrasound and compounds that become cytotoxic when exposed to ultrasound. It is similar to photodynamic therapy (PDT) in its advantages of specificity and low toxicity, but unlike light, sound is able to penetrate far into the body to reach interior tumours. A novel system is presented for the treatment of solutions and cell cultures with ultrasound, as is a particle counter-based survival assay for cells treated with ultrasound. The survival characteristics of human promyelocytic leukemia cells treated using this system were examined and a statistical model is presented to describe the survival behaviour. The model was extended for use in mechanistic studies of sonosensitizers, and was used to examine the sonodynamic mechanisms of three common drug solvents. A panel of compounds originally examined for photosensitizing activity was screened for sonosensitizing activity and the results of this screen are presented.

For my grandmother, Anna MacGregor.

I wish to thank my Mom and Dad for their continued insistence that I finish this thesis, my supervisory committee, Drs Miller, Wiebe and Lown, for their support of my experimental work and indulgence of my theoretical work, and my statistical advisor, Dr Doug Wiens, without whom I would have had no idea how to justify my theories.

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ATX-70	2,4-*Bis*(1-decyloxyethyl)-Ga(III)-1,3,5,8- tetramethylporphryin-6,7-dipropionyl diaspartic acid
cm	Centimetre
°C	Degrees Celsius
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
EPR	Electron paramagnetic resonance
FAD104	3′-Deamino-2′-fluoro-3′-hydroxydoxorubicin-14-pimelate
g	gramme
Hz	Hertz, cycles per second
kHz	Kilohertz
K	Degrees Kelvin
mL	Millilitre
µL	Microlitre
MHz	Megahertz
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDT	Photodynamic therapy
PEG	Polyethyleneglycol
Ph-A	Pheophorbide A
RPMI	Roswell Park Memorial Institute
SDT	Sonodynamic therapy
TMPone	2,2,6,6-Tetramethyl-4-piperidone
W	Watt

CHAPTER 1

General Introduction: Sonodynamic cancer therapy and the physical, chemical and biological bases of sonotoxicity

1.1 Introduction

Traditional treatments for cancer include surgical removal of tumour tissue, chemotherapy using compounds that are toxic principally to rapidly growing cells, and radiation therapy, where ionizing radiation is directed principally to the tumour and disrupts the replication of its genetic material. Surgery is the primary treatment modality for nonhematologic cancers, and is often used as a palliative measure to remove blockages of airways and blood vessels.

For localized tumours, the initial surgical resection is often the best opportunity for complete eradication of the cancer. Surgery, though, is an invasive treatment, and the sites of incision are subject to infection. Further, since the object of cancer surgery is the removal all cancerous cells, it is necessary to avoid seeding the operative field with cells from the resected tumour since these can produce further lesions. Since surgery is principally effective in the treatment of localized disease, the most frequent reason for the failure of surgery in the treatment of cancer is the presence of metastases outside the operative field (Capizzi, 1990).

Radiotherapy involves the use of ionizing radiation which is directed through the tumour volume. It is often curative for lymphomas and carcinomas of the head and neck, and has palliative use in reducing obstructions of the lungs, esophagus, and urinary and gastrointestinal (GI) tracts. Morbidity caused by radiotherapy is related to damage of normal tissues surrounding the tumour

(Tannock and Hill, 1992; Bentzen and Overgaard, 1997), and typical early side effects are bone marrow suppression, sterility, and radiation pneumonitis. In those cases where the initial disease is cured, leukemogenesis and carcinogenesis are side effects which may appear years later (Capizzi, 1990; Bentzen and Overgaard, 1997).

Chemotherapy involves the use of cytotoxic drugs to arrest or shrink tumours, and is used most frequently in the treatment of widely disseminated disease (Tannock and Hill, 1992). Chemotherapy can be curative for some diseases, such as Hodgkin's disease and acute lymphocytic leukemia of childhood, but is not usually used for palliative treatment due to its severe toxicity to normal tissue (Capizzi, 1990). Chemotherapeutic agents are selectively toxic to rapidly proliferating cells. Because of this, they are toxic to tumour cells but also show significant toxicity to the constantly renewed tissues of the bone marrow and GI epithelium (Tannock and Hill, 1992). Toxicity to the GI epithelium results in severe nutritional deficit, while toxicity to the bone marrow results in immune and hematopoietic suppression which leaves patients subject to opportunistic infection. Such toxic effects cause significant morbidity in patients receiving chemotherapy (Capizzi, 1990).

Compounds have been identified which sensitize tumours to exposure to visible light (Raab, 1900; von Tappeiner, 1900, Dougherty, 1974). These photosensitizing compounds, relatively nontoxic by themselves, become potent

toxins when exposed to light, damaging tissues by generating reactive oxygen species and free radicals (Pooler and Valenzeno, 1981; Ochsner, 1997). Certain porphyrin derivatives have been shown to possess such photosensitizing activity, and this property has been exploited to develop photosensitizer-based treatments for superficial tumours. Treatments of this kind are referred to as *photodynamic therapy*, or PDT. PDT has been effective in the treatment of superficial tumours of the skin and digestive tract. Photodynamic treatment of early-stage esophageal cancers has often been curative, and PDT is also effective in the palliation of advanced neoplastic disease (Marcus, 1992). It is a relatively noninvasive procedure, and has limited side effects beyond acute cutaneous photosensitivity for several weeks following treatment (Estey *et al.*, 1996). The principal limitation of PDT is the effective depth to which visible light can penetrate tissue. This is usually limited to approximately 5-7 mm, and prevents the use of PDT on deep-seated tumours. Though interstitial illumination systems have been devised to irradiate such tumours (Dougherty *et al.*, 1981; Arnfield *et al.*, 1986), such systems reduce the noninvasive nature of the treatment.

In the late 1980's, it was noted that certain photosensitizers and some chemotherapeutic drugs possessed another property, perhaps related to photosensitization: the ability to sensitize cells to ultrasound (Yumita *et al.*, 1989). This property has also been observed for some differentiation-inducing organic solvents (Jeffers *et al.*, 1995). Such cytotoxicity in connection with

ultrasound exposure has been termed *sonodynamic therapy*, or SDT (Umemura *et al.*, 1989). The theoretical advantage of SDT over PDT in the treatment of neoplastic disease is the potential for ultrasound to penetrate far into the body to activate sensitizers for the treatment of interior tumours (Meunier *et al.*, 1996). Targeting the sensitizing compound preferentially to the tumour mass may increase the specificity of PDT (Miller and Lown, 1997). This may be possible by enclosing sensitizers in liposomes (Richter *et al.*, 1993; van Leengoed *et al.*, 1994; Bachor *et al.*, 1995), preparing them as immunoconjugates (Mew *et al.*, 1983; Thorpe *et al.*, 1995; Trail *et al.*, 1995), or, combining the two approaches, by delivering them via immunotargeted liposomes (Morgan *et al.*, 1989; Morgan *et al.*, 1994). Equivalent approaches for SDT would give the treatment dual specificity, first from the preferential accumulation of the sensitizer within the tumour, and second from the activating energy focused principally on the tumour volume (Singh *et al.*, 1996).

1.2 Physical Effects of Ultrasound

Ultrasound consists of mechanical compression waves which propagate through various media. The only difference between ultrasound and ordinary sound is the frequency range— ultrasound frequencies are higher than those of audible sound and range from 1.8×10^4 to greater than 10^7 cycles per second, or Hertz (Hz). Like other forms of wave energy, ultrasound can be focused, reflected and refracted, and these properties form the basis for its use in imaging procedures (ultrasonography). As ultrasound is absorbed during

propagation through a medium, it deposits energy in the form of heat. The heating which it induces is exploited in physiotherapeutic applications of ultrasound (Lehmann *et al.*, 1966; Patrick, 1966).

Beyond these basic properties shared with other waves, ultrasound exhibits unique properties when propagating through water. Above a certain threshold intensity, propagation of ultrasonic waves through water elicits an effect termed 'cavitation' (Rayleigh, 1917; Connolly and Fox, 1954). Cavitation involves the formation of small bubbles or 'cavities' in the water during the rarefaction half of the wave cycle, followed by the collapse of these bubbles during the compression half of the cycle (Putterman, 1995). Cavities focus the energy of the incident ultrasonic radiation by many orders of magnitude (Hiller *et al.*, 1992). The consequence is that regions of cavitation in water are sites of extremely high temperature and pressure. Estimates of the temperatures generated in a collapsing cavity range from 5000K to 10^6K (Suslick *et al.* 1986; Flint and Suslick, 1991; Misik and Riesz, 1995; Kaiser, 1995). The magnitude of these temperatures has generated considerable interest among atomic physicists because they are great enough to induce hot fusion of deuterium nuclei at much lower cost than laser-based fusion systems (Pool, 1994).

In addition to inducing local regions of high temperature and pressure, cavitating water has powerful erosive effects on solid and semisolid substances due to the collapse of cavities against their surfaces. Cavitational

collapse produces small jets of water. When these jets impinge upon a solid surface they produce small pits, and constant exposure to these jets results in substantial erosion of the surface. Indeed, it was investigation of the erosion of the propellers of the HMS *Daring* by Sir John Thorneycroft and Sidney Barby that led to the discovery of cavitation at the end of the Nineteenth Century (Mason, 1991).

A further effect of cavitation is the emission of light from a cavitating medium, a phenomenon termed 'sonoluminescence' (SL) (Crum and Roy, 1994). Sonoluminescence occurs in two forms. Multiple bubble sonoluminescence (MBSL) is the result of spontaneous formation of many heterogeneous cavities throughout the medium and their asynchronous emission of photons. Consequently, the emission spectrum of MBSL is poorly defined. Single bubble sonoluminescence (SBSL) results from the artificial introduction of a single bubble into the insonation medium and the trapping of this bubble by focused incident sound (Gaitan and Crum, 1990). Single bubble SL produces a very bright luminescence whose spectrum has been well defined for wavelengths down to approximately 200nm, the ultraviolet cutoff frequency for transmission of light through water. Sonoluminescent photons are emitted principally in the blue and ultraviolet regions of the spectrum and can be modeled well by a

blackbody emission* curve. Sonoluminescence therefore appears to be the result of blackbody photon emission from hot cavities (Hiller *et al.*, 1992).

1.3 Chemical Effects of Ultrasound

Chemical effects of ultrasound are more limited and are related to the heat and erosion associated with cavitation. It has been known since the 1920's that exposure of certain reactive mixtures to cavitating water increases the rate of chemical reaction (Mason, 1991). The localized heat and pressure associated with cavitation cause the pyrolysis of the reagents and solvents, resulting in the formation of radical species in the cavitation region (Crum and Roy, 1994; Martí *et al.*, 1996; Misik and Riesz, 1996). The very reactive pyrolytic products are responsible for the observed increase in reaction rates. Ultrasonic erosion has been exploited to improve the efficiency of metal catalysts by increasing their available surface area, and has also been used to improve the rate of Grignard syntheses by forcing magnesium particles into suspension (Mason, 1991).

1.4 Biological Effects of Ultrasound

The biological effects of exposure to ultrasound are the result of its physical and chemical effects. The most obvious biological effects of ultrasonic treatment stem from heating of the medium through which it passes. Such heating is exploited during physiotherapy to help heal injured tissues

* Blackbody emission is the electromagnetic emission characteristic of hot matter in cases where the radiative energy is able to equilibrate with the emitting body through absorption and re-emission events before its escape. The most familiar example of blackbody emission is the glowing filament of an incandescent lightbulb.

(Lehmann *et al.*, 1967; Patrick, 1966), and has been investigated as a possible modality for tumour treatment. This is due to the sensitivity of many tumours to hyperthermia, a state in which tissue temperatures are elevated above 42°C (Doss and McCabe, 1976; Marmor *et al.*, 1979; Sculier and Klastersky, 1981; Bleehen, 1982; Hynynen and Lulu, 1990). Ultrasound has also been used in combination with radiation therapy to improve treatment response *in vivo* compared to radiotherapy alone (Clarke *et al.*, 1970; Repacholi *et al.*, 1971; Mitsumori *et al.*, 1996). A principal danger in the use of ultrasound for therapeutic purposes is the formation of 'hotspots' due to regions of constructive interference and preferential absorption of ultrasonic energy by bone regions with low curvature radii[†] (Lehmann *et al.*, 1967; Linke *et al.*, 1973). These hotspots can cause serious damage to nearby tissues (Hill, 1968; Bruno *et al.*, 1998).

Ultrasound can induce cavitation in living tissue. Though this has been known for the frequencies used in physiotherapy (1- 3MHz), the danger associated with cavitation induced by diagnostic ultrasound (~10MHz) has only recently come under investigation (Suhr *et al.*, 1996). Because certain soft-tissue interfaces reflect ultrasound poorly, contrast agents have been developed to improve the quality of ultrasonic imaging. These agents may act as nuclei for cavitation, which may then damage cells by free-radical production or erosion of the cell membrane (Mornstein, 1997). It has also been noted that ultrasonic shear forces are able to disrupt cellular membranes (Worthington *et al.*, 1997) and to fragment cellular DNA (Galperin-Lemaitre *et al.*, 1975).

[†] Low curvature radii are associated with tightly curved regions at the ends of bones, while larger curvature radii are associated with longer, straighter regions.

1.5 Sonodynamic Therapy

Another effect of ultrasound on living tissue appears when it is used in conjunction with some photosensitizers and certain other compounds. Treatment of cells with hematoporphyrin (Hp) has been observed to increase cells' sensitivity to ultrasonic exposure (Umemura *et al.*, 1989). This may be related to photodynamic processes since sonoluminescence produced in saline solution has been observed to have spectral components that can excite Hp molecules to produce reactive species. These may then attack the cell membrane or other targets in the cell in a manner similar to that observed for photodynamic activity (Umemura *et al.*, 1990). In spite of the intuitive nature of this parallel with PDT, Kessel *et al.*, in comparing the mechanisms of PDT and SDT, found no relation between the photo- and sonodynamic activity of any given porphyrin (Kessel *et al.*, 1994).

The combination of ultrasound with certain antitumour drugs has been studied since the 1980's as a means of increasing the efficacy of chemotherapy. Since early studies concerned drugs that had antitumour effects by themselves, it was difficult to determine if the increased toxicity of the combination therapy were due to additive or synergistic effects (Umemura *et al.*, 1990). More recently it has been found that Hp, a photosensitizing compound with no intrinsic cytotoxic activity, increases the sensitivity of cells to ultrasound. In experiments in which mouse sarcoma 180 or rat ascites hepatoma 130 cells were exposed to ultrasound up to an intensity of $2W/cm^2$ in the presence or absence of Hp, Hp alone showed no cytotoxic effect at concentrations up to 50μg/mL. Ultrasound alone damaged up to 50% of cells. In the presence of Hp, however, ultrasound damaged between 95% and 99% of the cells. These results support the

concept that Hp and ultrasound do demonstrate synergistic cytotoxicity (Yumita *et al.*, 1989).

An *in vivo* study in mice also demonstrated that treatment with ultrasound and 50mg/kg Hp caused complete inhibition of tumour growth, while treatment with ultrasound alone had only a small inhibitory effect. Antitumour effects in mice implanted subcutaneously with sarcoma 180 in the left dorsal scapula region were evaluated by measuring the tumour size and weight. Where Hp alone showed no antitumour effect and ultrasound alone showed a slight antitumour effect, the combined treatment with ultrasound and Hp showed marked synergistic effects on sarcoma 180 (Yumita *et al.*, 1990).

Umemura *et al.* were the first to study the molecular consequences of sonodynamic activation, examining the mechanism of cell damage by ultrasound in combination with Hp. Sarcoma 180 cell suspensions were exposed to ultrasound in the presence and absence of 50μg/mL Hp, with and without the active oxygen scavengers mannitol and histidine. The sonodynamic toxicity of Hp was suppressed upon addition of histidine, which reacts with singlet oxygen, but not mannitol, which reacts with hydroxyl radicals. The cytotoxicity was also found to double in rate upon substitution of deuterium oxide for water, a manipulation which increases the yield of singlet oxygen. These results suggest that cell damage enhancement is likely mediated by singlet oxygen generated by ultrasound activated Hp (Umemura *et al.*, 1990).

This line of inquiry was continued by Yumita *et al.*, who studied sonodynamically generated active species using electron paramagnetic resonance (EPR) spectroscopy. Nitroxide production from the reaction of

TMPone (2,2,6,6-tetramethyl-4-piperidone) with active oxygen was significantly higher in the presence of Hp than in its absence. It was found to be substantially reduced by the presence of sodium azide or histidine— both singlet oxygen scavengers— in solution with Hp. Yumita *et al.* also provided chemical confirmation of the earlier results of Umemura *et al.*, whose biological assay had implicated singlet oxygen as a mediator of Hp's cytotoxic effect. Replacement of the water solvent with deuterium oxide doubled the nitroxide production from TMPone in the presence of Hp, while the production without Hp increased very little. Nitroxide production from TMPone in the presence of Hp was not reduced by the addition of mannitol and no nitroxide was produced in nitrogen-saturated solutions, which limit production of singlet oxygen. The effects of mannitol and nitrogen provide further evidence that hydroxyl radicals are a negligible component of the active oxygen species produced by ultrasound in the presence of Hp. Together, these results suggest that insonation induces significant and almost exclusive production of singlet molecular oxygen in aqueous Hp solutions (Yumita *et al.*, 1994).

The first examination of cellular effects of free radicals produced by ultrasound concerned hemolysis in a cavitating medium. Cavitation was later found necessary for the induction of sonodynamic effects (Kessel *et al.*, 1994). Kondo *et al.* studied hemolysis, membrane fluidity, membrane permeability, and membrane deformability following insonation of erythrocyte suspensions in the absence of any sonosensitizer. Free radical formation was determined by EPR. Hemolysis and membrane alteration of erythrocytes were examined using insonation conditions designed to distinguish between cavitation with or without free radical formation, and using γ-irradiation to generate large quantities of free radicals. Free radical formation by ultrasound was observed

in the presence of argon as the ambient gas, while substitution of N_2O for argon eliminated free radical production. Hemolysis induced by ultrasound was similar in the presence of both gases. After insonation in both conditions, the permeability, fluidity, and deformability of the unlysed erythrocytes were the same as in uninsonated control cells. Following gamma irradiation, a positive control for the effect of large quantities of free radicals, the hemolysis behavior was quite different from that after insonation, and the membrane properties were significantly changed. In this work, membrane properties were found to be the same under conditions where free radicals can be formed by ultrasonic action and under conditions where they cannot. This suggests that the action of free radicals in hemolysis induced by insonation alone is minimal and that cell lysis is due principally to mechanical stress arising from ultrasonic exposure (Kondo *et al.*, 1988).

The effects on cells of the intermediate species produced by insonation in the presence of Hp were examined by Yumita *et al.* Membrane damage to rat erythrocytes due to lipid peroxidation after insonation with Hp was assayed by exposing suspensions of erythrocyte ghosts and intact erythrocytes to ultrasound in the presence and absence of 80μM Hp. Hp increased the level of ultrasound-induced lipid peroxidation by up to five times, while Hp treatment without insonation showed no peroxidation. The rates of peroxidation and of hemolysis were related to Hp concentration, to acoustic conditions, and to chemical conditions such as the presence or absence of active oxygen scavengers. Relating this to their 1994 investigation of the sonochemical action of Hp, they found that sonodynamically induced lipid peroxidation in the presence of Hp doubled upon the substitution of deuterium oxide for water. Peroxidation was significantly reduced by histidine, by sodium azide, and by

substitution of nitrogen for air as the ambient gas. Addition of superoxide dismutase and mannitol did not inhibit peroxidation. These results are consistent with the hypothesis that lipid peroxidation by singlet oxygen is the primary mechanism of sonodynamic enhancement of hemolysis by Hp (Yumita *et al.*, 1996).

The limited effect of radical species other than singlet oxygen in sonodynamic sensitization was investigated by Worthington *et al.* Immediate cell lysis and loss of cell colony forming ability were measured *in vitro* for insonated Chinese hamster ovary cells in the absence of sensitizer. A general correlation was observed between cavitation, free radical production, and cytotoxicity, but the yield of free radicals was too small to explain the extent of cell killing observed. This result tends to confirm that cytotoxicity is not related to attack by extracellular free radicals. Immediate cell lysis, and consequent loss of colony forming ability, was found to be closely linked to cavitation. Cavitation is known to produce shear forces that disrupt cellular membranes, and this may be enhanced by damage from singlet molecular oxygen (Worthington *et al.*, 1997).

The general effect of ultrasonic destruction of cells, with or without sensitizer, was also examined by Kessel *et al.* This work showed that viability after insonation of murine leukemia L1210 cells containing intracellular porphyrins was not different from insonated controls not treated with porphyrins. Loss of viability was related to inhibition of amino acid transport and cell fragmentation, which implicates disruption of the cell membrane as the principal cytotoxic effect. They further found that the porphyrins tested had sonodynamic activity *in vitro* only when present in the incubation medium (Kessel *et al.*, 1994).

Tata and Dunn (1991) examined the kinetics of ultrasound mediated cell death in sarcoma 180 cells. They identified two subpopulations with different sensitivities to ultrasonic destruction, and found the survival of each subpopulation to obey first-order kinetics. The rate of cellular destruction was proportional to the intensity of the applied ultrasound and when tested in the presence of Hp was further found to be a function of the sensitizer concentration (Tata and Dunn, 1991).

Kessel *et al.* also compared effects of ultrasound-induced *vs* photodynamic cytotoxicity in cell culture. Photodynamic effects mediated by mesoporphyrin were found to cause a delayed toxic reaction; the 'shoulder' found on the dose-response curve indicated the ability of cells to repair limited photodynamic damage induced by Hp. In contrast, ultrasound-induced loss of viability resulted from rapid cell destruction and was proportional to the time of insonation. Cells treated with mesoporphyrin followed by irradiation with 500nm light were found to be significantly more sensitive to ultrasonic destruction. The clonogenicity of surviving cells was not found to be different from that of untreated control cells. Photodamage after exposure to ultrasound decreased the viability of cells which had survived ultrasonic treatment to a lesser extent than did insonation following photodamage (Kessel *et al.*, 1995). These results indicate that photodynamic therapy with mesoporphyrin sensitizer weakens cells to ultrasonic destruction, and that irradiation and then insonation of photosensitizer-treated tissue may be useful in cancer therapy.

1.6 New Sonosensitizers

The first compounds to have identifiable sonotoxic effects were certain existing chemotherapeutic agents (Umemura *et al.*, 1990). In their investigation of

potentiation of chemotherapeutic cell killing by low-level ultrasound, Harrison *et al.* found synergistic effects of doxorubicin and diaziquone with tone-burst and pulsed ultrasound[§]. They observed significant ultrasound-induced increases in drug cytotoxicity *in vitro* in two of the three cell lines they used. Testing of the sonodynamic activity of these drugs *in vivo* showed significant antitumour effect as measured by volume reduction in uterine cervical squamous cell carcinomas in Syrian hamsters (Harrison *et al.*, 1991). The molecular basis of the sonodynamic effect of doxorubicin was also examined by Umemura *et al.*, who found that ultrasound-induced cell damage and nitroxide production with TMPone were closely related, and that both effects were inhibited by the addition of histidine. These results are consistent with a sonodynamic mechanism that is related to the ultrasound-induced production of active oxygen species and similar to that observed for Hp (Umemura *et al.*, 1997).

The sonodynamic effect of a compound structurally related to doxorubicin, the fluorine-containing anthracycline derivative FAD104 (3′-deamino-2′-fluoro-3′-hydroxydoxorubicin-14-pimelate) was investigated *in vitro* by Yumita *et al.* Studies of sarcoma 180 cells insonated in the presence and absence of FAD104 demonstrated that the rate of cell damage doubled in the presence of 80μM FAD104, while no cell damage was observed with FAD104 alone. As with doxorubicin and Hp, the synergy between ultrasound and FAD104 was significantly inhibited by histidine, again suggesting a sonotoxic mechanism related to the production of reactive oxygen species (Yumita *et al.*, 1998).

[§] A tone-burst refers to a signal of short duration that has many component frequencies at its start, most of which rapidly die off to give one major frequency by the end of the burst. A pulse is a short signal of constant frequency composition through its duration.

Pheophorbide A (Ph-A) has also been noted to possess synergistic cytotoxic effects in combination with ultrasound. Umemura *et al.* investigated the sonodynamic effect of Ph-A *in vitro* and *in vivo* on sarcoma 180 cells. The presence of 80μM Ph-A was found to double the rate of ultrasound-induced cell damage. This was significantly inhibited by histidine, which suggests that this effect too was mediated by sonodynamically generated oxygen species. Studies in mice where 5mg/kg Ph-A was administered before insonation, showed that ultrasound treatment completely inhibited tumour growth at an intensity at which ultrasound alone showed little antitumour effect (Umemura *et al.*, 1996: *Sonodynamically Enhanced Effect of Pheophorbide A*).

Some polar solvents have been investigated as anticancer drugs due to their ability to induce differentiation in some tumour cell lines, but their clinical utility is precluded by hepatotoxic side effects. Jeffers *et al.* investigated the effects of DMSO, DMF and MMF in combination with ultrasound on HL-60 human promyelocytic leukemia cells. In conditions where acoustic cavitation was facilitated by the presence of albumin-stabilized microbubbles, all three solvents were noted to have synergistic effects in combination with ultrasound. It was also found that microbubbles are necessary for the cytotoxic synergy between the solvents and ultrasound, suggesting that the mechanism may be dependent upon acoustic cavitation. They also speculate that the microbubbles used in the study, similar to contrast agents used in ultrasonography, may provide a ready means of facilitating ultrasonic cavitation for the stimulation of sonodynamic agents. This may enable the use of lower ultrasonic intensities for therapeutic purposes (Jeffers *et al.*, 1995). Misik and Riesz later investigated these solvents with EPR spin-trapping studies which showed the presence of solvent-derived radical adducts following insonation. They

speculated that the sonodynamic effect observed by Jeffers *et al.* is caused by these radical species, which are longer-lived than the •OH and •H radicals also produced during insonation (Misik and Riesz, 1996).

The most promising new sonosensitizer is a gallium-porphyrin complex, ATX-70 (2,4-*bis*(1-decyloxyethyl)-Ga(III)-1,3,5,8- tetramethylporphryin-6,7-dipropionyl diaspartic acid). Enhancement of ultrasound-induced cell damage *in vitro* by ATX-70 was investigated by Umemura *et al.* Where 80μM Hp was found to double the rate of ultrasound-induced damage to sarcoma 180 cells, ATX-70 at the same concentration increased the rate of damage in excess of four times. Addition of histidine was found to inhibit the sonodynamic effect, while addition of mannitol had no effect. This indicates that singlet molecular oxygen may be the principal mediator of the observed sonodynamic toxicity. EPR studies of insonated solutions of ATX-70 showed that the reaction of TMPone with active oxygen species produced levels of nitroxide 2.5 times greater than those produced by solutions containing Hp. Singlet oxygen production was confirmed by the bleaching of N,N-dimethyl-4-nitrosoaniline in the presence of imidazole. Comparable to the difference in nitroxide production, ultrasound-induced bleaching was three times as great in the presence of ATX-70 as in the presence of Hp at the same concentration (Umemura *et al.*, 1993).

The sonodynamic effect of ATX-70 *in vivo* was studied by Yumita *et al.* in mice implanted subcutaneously with colon 26 carcinoma in the left dorsal scapula region. Treatment with ATX-70 alone had no effect, while treatment with ultrasound in the absence of sensitizer had a slight antitumour effect. The antitumour effect was found to increase in a dose-dependent manner after administration of ATX-70. Tumours treated with ATX-70 in combination with

ultrasound were found to be one-third as large in diameter as untreated tumours three days after treatment (Yumita *et al.*, 1996). Control mice treated with ultrasound alone or ATX-70 alone showed no tumour necrosis, while those treated with ultrasound and ATX-70 together showed substantial tumour destruction (Yumita *et al.*, 1997). A subsequent *in vivo* study of the sonodynamic effect of ATX-70 with high-intensity ultrasound showed similar results. At doses above 2.5mg/kg, ATX-70 showed significant antitumour effect at an average acoustic intensity of 12 W/cm^2 in rats bearing Walker 256 tumours. The investigation also showed no sonodynamic effect at ultrasound intensities below 8W/cm^2 at any sensitizer concentration, or at sensitizer doses below 1.0 mg/kg at ultrasound intensities up to 40W/cm^2 (Sasaki *et al.*, 1998).

Considerable effort has been exerted in the development of SDT as a viable therapeutic modality. Hp and related compounds have been studied extensively. Their sonodynamic effect has been established and several lines of evidence point to mediation of this effect by singlet molecular oxygen. The range of possible sonodynamic agents, while still composed principally of porphyrin derivatives, has expanded substantially. It now includes other chemotherapeutic agents such as doxorubicin derivatives, certain solvents such as DMSO, DMF and MMF (which are too toxic to be used clinically) and other compounds, such as pheophorbide A. The sonodynamic activity of these new sensitizers has also been related to the generation of singlet oxygen. The most promising of these new sonodynamic agents is ATX-70, whose sonodynamic effect is twice as great as that of Hp.

1.7 Importance of SDT

While these discoveries represent significant advances, two serious deficiencies remain in the development of experimental SDT. A substantial problem is the lack of sonodynamic agents with favourable clinical properties. Porphyrins are known to cause significant cutaneous photosensitivity (Estey *et al.*, 1996), doxorubicin is cardiotoxic (Myers *et al.*, 1976), and DMSO, DMF and MMF are hepatotoxic (Misik and Riesz, 1996). New sensitizers with better sonodynamic properties which have milder side effects and which are rapidly cleared would greatly improve the clinical application of SDT. A further problem is the lack of standardization in the conditions used for evaluating sonodynamic agents. Potential sonodynamic agents have been tested following exposure to ultrasound intensities ranging from 0.25W/cm^2 to 40W/cm^2, and frequencies from 500kHz to 1MHz (Harrison *et al.*, 1991; Sasaki *et al.*, 1998). Further, though *in vivo* use would seem to require greater energies due to roughly isotropic dissipation of the ultrasonic energy, little effort has been made to compare experimental conditions *in vitro* with those *in vivo*. Where one group will find evidence of sonodynamic effect, different investigators do not under apparently similar conditions. Development of a standard insonation and assay system compatible with clinical use will permit a more rigorous assessment of the sonodynamic effects of current and future sensitizers.

The importance of SDT lies ultimately in its similarity to PDT, an elegant and effective tumour treatment whose success is due to the use of light and drug in combination— two treatment elements, neither of which has toxic effects by itself (Marcus, 1992). PDT has mild side effects, destroys relatively little healthy tissue, and new photosensitizers with better therapeutic indices and improved clinical properties are being developed.

The principal impetus for the development of SDT has been improvement upon PDT's dosimetric shortcomings. PDT is now restricted to use on superficial tumours. Its use on tumours deep within the body requires interstitial irradiation that increases the complexity of the treatment and compromises its noninvasive nature. SDT provides a means to reach such tumours, since ultrasound propagates easily through several centimeters of tissue, and like light, can be focused principally on the tumour mass where it activates the sonosensitizing compound. Targeted SDT offers the possibility of improving the tolerance of this therapy by further restricting its effects to the target tissue.

1.8 Thesis Objectives

There are three elements to the work described in this thesis. First is the development of an *in vitro* insonation system based upon a physiotherapy ultrasound source. A cytotoxicity assay using a particle counter was then developed for use with this insonation system. This experimental system permits substantial simplification and standardization of SDT research. It demonstrates the utility of a commercial ultrasound source for SDT research, and may lead to the adoption of such an apparatus as the standard for SDT experiments. The thorough characterization of a complete model system also facilitates comparison of SDT experiments among research groups.

Second, the ultrasound dose-response for human promyelocytic leukemia HL-60 cells was investigated using this system, and a model for the kinetics of HL-60 cell survival in the presence of ultrasound was proposed. The biological meaning of this model was investigated, and it was extended to permit a new distinction between sonodynamic sensitizing agents and sonosensitizers with no dynamic effects. This provides a novel means of analyzing the mechanism

of action of sonosensitizers and grouping them into different classes based on a relatively simple statistical test. The model was then applied to survival data for certain solvent sensitizers to demonstrate that their mechanism of action may be different from the mechanisms of clinically desirable sonosensitizers. The final portion of the research involved the screening of three panels of photosensitizers for sonosensitizing activity. This experimental work extends the range of known sonosensitizing compounds to two further classes of compounds which are known to have much less severe clinical side effects than those compounds currently under investigation as sonosensitizers. Since members of these classes of compounds are known to possess photodynamic activity, the discovery of sonosensitizers among these compounds provides further heuristic evidence of a link between SDT and PDT.

Chapter 2

General Methods

2.1 Chemicals

Table 2.1. General laboratory reagents used in experiments supporting this thesis.

Chemical	Source
Agar	Difco (Detroit, MI)
Dimethylsulphoxide	Sigma-Aldrich Canada (Oakville, ON)
Ethanol	Commercial Alcohols (Winnipeg, MB)
Fetal bovine serum	CanSera (Rexdale, ON)
Fisher's medium	Gibco (Grand Island, NY)
Hydrochloric acid	Fisher Scientific (Nepean, ON)
Isoton II counting diluent	Beckman-Coulter (Fullerton, CA)
Penicillin-streptomycin-neomycin antibiotic	Gibco (Grand Island, NY)
Propidium iodide	Sigma-Aldrich Canada (Oakville, ON)
RNase A	Sigma-Aldrich Canada (Oakville, ON)
RPMI-1640 medium base with L-glutamine	Gibco (Grand Island, NY)
Sodium bicarbonate	BDH (Toronto, ON)
Sodium hydroxide	BDH (Toronto, ON)
Triton X-100	BDH (Toronto, ON)

Table 2.2. Potential sonosensitizing compounds used in experiments supporting this thesis.

Sensitizer	**Source**
Porphyrin sensitizers:	
Hematoporphyrin dihydrochloride	Porphyrin Products (Logan, UT)
Photofrin II	QLT Phototherapeutics (Vancouver, BC)
Solvent sensitizers:	
Dimethylsulphoxide	Sigma-Aldrich Canada (Oakville, ON)
N,N-dimethylformamide	Aldrich Chemicals (Milwaukee, WI)
N-methylformamide	Aldrich Chemicals (Milwaukee, WI)
Polyethyleneglycol-300	Sigma-Aldrich Canada (Oakville, ON)
Diaminoanthraquinone sensitizers:	*Diaminoanthraquinone sensitizers were a gift of Dr J W Lown* **
A-2-I ($C_{30}H_{24}O_{10}N_2S_2Na_2$)	
A-6-II ($C_{20}H_{22}O_4N_2$)	
A-11-IV-B ($C_{26}H_{34}O_4N_2$)	
A-11-V-B ($C_{22}H_{26}O_4N_2$)	
A-13-II ($C_{24}H_{32}O_6N_4$)	
A-20-I ($C_{16}H_{13}O_4N$)	
A-20-II ($C_{16}H_{14}O_4N_2$)	
A-31-II ($C_{22}H_{26}O_8N_2$)	
A-41-I ($C_{24}H_{30}O_6N_2S_2$)	

Continued...

** Structural formulae for the diaminoanthraquinone compounds are given with the sonotoxicity data of these compounds in Chapter 6.

Table 2.2. *Continued*

A-52-III ($C_{22}H_{26}O_6N_2S_2$)	
A-55-II	
A-61-II	
DND sensitizers:	*DND sensitizers were a gift of Dr J W Lown* **
DAO-5 ($C_{30}H_{26}O_7N_2$)	
DAO-9 ($C_{16}H_{20}O_2N_2$)	
DND-64 ($C_{24}H_{24}ON_4$)	
DND-69 ($C_{24}H_{22}O_2N_4$)	
DND-71 ($C_{22}H_{20}O_1N_4$)	
RH-700 ($C_{28}H_{26}O_5N_2ClF_3$)	
RH-800 ($C_{28}H_{26}O_5N_3Cl$)	
Perylenequinonoid sensitizers:	*Perylenequinonoid sensitizers were a gift of Dr J W Lown***
Cercosporin (CP) ($C_{29}H_{26}O_{10}$)	
CP-$Mg(OAc)_2$ ($C_{33}H_{28}O_{12}Mg$)	
DBHB ($C_{30}H_{23}O_9Br$)	
DMHB ($C_{32}H_{28}O_9$)	
DW-23 ($C_{30}H_{26}O_{12}$)	
DW-23- $Mg(OAc)_2$ ($C_{34}H_{30}O_{14}Mg$)	
HA (Hypocrellin A) ($C_{30}H_{26}O_{10}$)	
HA-$Mg(OAc)_2$ ($C_{34}H_{28}O_{12}Mg$)	
HB (Hypocrellin B) ($C_{30}H_{24}O_9$)	

Continued...

** Structural formulae for these compounds are given with their sonotoxicity data in Chapter 6.

Table 2.2. *Continued*

HBAM-R1 ($C_{42}H_{48}N_4O_9$)	
HBAM-R2 ($C_{42}H_{48}N_4O_9$)	
HBBA-R2 ($C_{36}H_{60}N_4O_7$)	
HBDD-R1 ($C_{42}H_{52}N_4O_7$)	
HBDP-R1 ($C_{40}H_{48}N_4O_7$)	
HBEA-R1 ($C_{34}H_{34}N_2O_9$)	
HBED ($C_{38}H_{32}N_8O_6$)	
HBIC ($C_{30}H_{22}O_9I_2$)	
HBMA-R2 ($C_{30}H_{33}N_3O_6$)	
HB-$Mg(OAc)_2$ Complex ($C_{34}H_{30}O_{13}$)	
JL-1-1 ($C_{30}H_{36}O_{12}$)	

2.2 Software

Analysis of data derived from experiments described in this thesis was performed using MS-Excel (Office 97) spreadsheet program (Microsoft Corp., Redmond, WA, 1997), SigmaPlot 3.02 (SPSS Inc., Chicago, IL, 1995), and S-Plus 4.5 (MathSoft Inc., Seattle, WA ,1998). Conversion of cell-count data to surviving fractions was performed using MS-Excel, and all graphs were produced using SigmaPlot. SigmaPlot was also used for making initial parameter estimates in model-fitting procedures; these estimates were then used in nonlinear regression procedures using the S-Plus program.

2.3 Ultrasound Source

The ultrasound generator consists of a physiotherapy ultrasound source (Excel Tech, Oakville, Ontario, Canada) which produces continuous-wave ultrasound

at selectable frequencies of 1MHz or 3MHz at intensities between 0.10W/cm^2 and 2.00W/cm^2. This device, shown in Plate 2.1, measures the acoustic coupling efficiency and estimates the energy transmitted to the insonation sample. It is customarily used to generate low intensity ultrasound for the heating of injured tissue during physiotherapy, and its wide use in this capacity makes it an economical apparatus for use in SDT studies. It is available from the manufacturer for approximately $1900.00.

Since the transducer is designed for use on solid tissue, it has a flat-surface stainless steel applicator. A Teflon™ cuff was fabricated for the applicator to form a vessel in which cell suspensions are insonated. The ultrasound source is autocalibrating and adjusts for the presence of the insonation cuff by measuring the acoustic impedance of the applicator with no acoustic load other than the insonation cuff.

The ultrasound generator detects the presence of the applicator in its mounting bracket during calibration, and will not autocalibrate unless the applicator breaks the infrared detector beam in the mounting area. Since the insonation cuff does not permit the applicator to be placed in the bracket, to calibrate the generator a piece of opaque adhesive tape must be placed over the infrared detector near the bracket, and the sample cuff must be empty and clean. The tape must be removed before commencing insonation since the generator will not function properly with it in place.

The insonation period is measured using either the timer, which counts down in one-second increments, or the energy integrator which is connected to the timer. The energy readout counts upward from zero in increments of 0.001kJ; at

an ultrasonic intensity of 2.00W/cm^2, each 0.001kJ indicates an elapsed time of 0.1s. Using the energy readout, insonations may be timed reliably to 0.1s.

Insonations were carried out by cleaning the cuff with a solution of 70% ethanol in water and wiping with a low-lint tissue, loading the sample, selecting the ultrasonic intensity, and pressing the start button. Insonation is terminated by pressing the 'pause' button, recording the insonation time, pressing the 'stop' button, and draining and cleaning the insonation cuff.

Hematoporphyrin(IX) dihydrochloride (Hp), a known sonosensitizer (Umemura *et al.*, 1993), was used to evaluate the effectiveness of the insonation and assay systems. Survival curves were constructed to determine the optimum sensitizer concentration and insonation time.

2.4 Cells and Culture Conditions

Human acute promyelocytic leukemia HL-60 cells (ATCC No CCL-240) were a gift of Ms. Louise Enns (Department of Experimental Oncology, Cross Cancer Institute, Edmonton, Alberta). They were grown in RPMI 1640 cell culture medium supplemented with 7g/L of L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin-neomycin. The medium was buffered with $NaHCO_3$ to pH= 7.4 according to the supplier's instructions. Cells were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2, and maintained at cell densities between 2.0×10^5 and 1.0×10^6 cells/mL.

2.5 Insonation Procedures

All preparations for insonation experiments were carried out in a biosafety hood, as were the insonations. This was done to prevent contamination of the

cultures and to prevent the escape of biohazardous aerosols from the insonation apparatus.

2.5.1 Ultrasonic Heating Tests

Ultrasound-induced temperature increase in the culture medium was evaluated as a function of insonation time at 1MHz and 2.00W/cm^2, the highest available intensity. For all tests, 10mL of medium was introduced into the cuff and its initial temperature was measured using a mercury thermometer mounted on a utility stand. The thermometer was raised out of the insonation cuff and the sample was insonated for intervals between one and five minutes. For all trials, the initial temperature of the medium was between 20°C and 22°C.

2.5.2 Preparation of Cells for Insonation

For insonations in the presence of solvents alone, cells were diluted to approximately 2.0×10^5 cells/mL with culture medium and the appropriate number of 10.5mL aliquots were prepared in 15mL centrifuge tubes. Solvent was then added to each centrifuge tube to give a concentration between 0.1% and 1.0% by volume. The tubes were then capped, inverted several times, and incubated in the dark at room temperature (20-22°C) for 30 minutes. Mechanical transfer loss during addition of the cells to the insonation apparatus, as determined by removing the contents of the insonation cuff using a 10mL graduated pipet, yielded 10.4mL of cell suspension per tube for insonation.

Preparations for insonation in the presence of Hp or photosensitizers were carried out in low-light conditions to avoid potential photosensitization. Cells

were harvested at a density of approximately 7×10^5 cells/mL. Sensitizer was weighed in a microcentrifuge tube. The quantity of compound used gave a final concentration between 0.3mM and 1mM in a volume of 13mL, depending upon the amount of compound available and the concentration at which the solution became saturated. On the day of the experiment, the sensitizer was dissolved in 70μL of tissue culture grade DMSO to facilitate its subsequent dissolution into the cell suspension. Cells were then resuspended and aliquoted to Petri plates, the first plate receiving 13mL and subsequent plates receiving 11.7mL. A few milliliters of cell suspension were withdrawn from the first plate into a pipet and the solution of sensitizer in DMSO was poured into the first plate. The microcentrifuge tube was then rinsed with cell suspension from the pipet to ensure quantitative addition of the DMSO. The cell suspension was mixed gently by drawing it into a pipet and expelling it several times, and, with a new pipet, 1.3mL from the first plate was added to the second plate and mixed gently. This was repeated for subsequent plates, and 1.3mL of cell suspension were withdrawn from the last plate and discarded to yield a final volume of 11.7mL for all plates. DMSO concentration was equalized by adding 56.7μL of DMSO to the second plate, 62.4μL of DMSO to the third plate, 62.9μL of DMSO to the fourth plate and 63μL of DMSO to subsequent plates.

The plates were incubated for four hours in the same conditions under which the cells were grown. This procedure gives a series of plates containing 11.7mL of cells with a constant concentration of DMSO and a sensitizer concentration decreasing by factors of ten. Mechanical transfer loss in adding the cells to the insonation apparatus, measured as described above, yielded 10.4mL of cell suspension from each plate for insonation.

Each set of samples included an internal negative control in which cells were treated only with DMSO at the same concentration as the other samples, and an internal positive control of 1mM Hp, prepared in the same manner as for the other sensitizer-treated samples. The average surviving fraction from the Hp control tests was computed and used for comparison with data from the screen. This average fraction and its 95% confidence interval are indicated on each survival curve for the compounds screened for sonosensitizing activity as described in Chapter 6.

2.5.3 Insonation of Cell Cultures

Cell suspensions treated with solvents only were insonated immediately following incubation, while cells treated with Hp or photosensitizers were allowed to equilibrate to room temperature for 30 minutes in the laminar flow hood in subdued light. Insonations were begun at room temperature to avoid hyperthermic conditions which may arise from the heating that accompanies ultrasonic exposure (see Section 3.2.2).

Each sample was gently resuspended and poured directly from the centrifuge tube or Petri plate into the insonation cuff where a 400μL aliquot was withdrawn and counted to estimate the initial cell density. Since 10.4mL of sample was aliquoted for each insonation, the total volume insonated was always 10.0mL. The sample was then insonated at an ultrasound intensity of 2.00W/cm^2 for the appropriate time, after which a second 400μL aliquot was withdrawn and counted to estimate the density of surviving cells. The counting procedure is outlined in Section 2.6.1.

Insonations were timed using the energy readout of the ultrasound generator as described in Section 2.3. For insonations used to generate survival curves as a function of time, samples were insonated for times varying from zero (no insonation) to 15 seconds. For insonations used to evaluate sonotoxicity of test compounds as a function of their concentration, all samples were insonated for 10 seconds. For experiments involving solvents only, four identical samples were prepared at each concentration and each sample was aliquoted and counted once. This was done to account for variation among samples. For experiments involving other sensitizers, only one sample was prepared at each concentration due to the small quantities of compound available, and each sample was aliquoted and then counted four times. Two samples containing counting diluent only were counted for each compound tested to measure background counts.

2.6 Cell Counting Procedures

Cells were counted using a Coulter Z1 particle counter (Beckman Coulter Inc, Fullerton, CA) fit with a 100μm aperture tube. The counter gate consists of a single size threshold above which counts are registered.

The size distribution of HL-60 cells was determined by constructing differential particle diameter curves. Counting a single suspension of cells above progressively higher diameters generates an integral diameter curve as shown in Figure 2.1. Taking the differences between successive counts produces a differential particle diameter curve which shows the actual size distribution of the cells. Such a curve is shown in Figure 2.2.

Subsequent to determining the cell size range, the counting gate was set to count particles of 9μm diameter and greater. An aliquot of 400μL of cell suspension was withdrawn directly from the culture flask or insonation cuff, and deposited in a cuvette containing 10mL of Isoton II counting diluent. The cuvette was then capped, inverted several times, uncapped, and placed on the counting stage. The counting gate was set and the start button was pressed. Between counts the counting probe was rinsed with a cuvette containing clean counting diluent. Each count was recorded and the sample was discarded.

2.7 Evaluation of the Insonation System

The insonation apparatus and survival assay were tested together using Hp. Survival curves were constructed as a function of Hp concentration at constant insonation time to determine the most practical sensitizer concentration as shown in Figure 2.3. No significant sonotoxicity, as determined by one-way ANOVA, was observed for Hp concentrations up to 0.1mM[††], and 1mM was selected as the concentration for positive control experiments. The difference in survival of Hp-treated and control cell suspensions was also plotted as a function of insonation time at an Hp concentration of 1mM to determine the optimum insonation time, as shown in Figure 2.4. Though the proportion of surviving Hp-treated cells relative to control cells decreases greatly with insonation time, the greatest absolute difference in survival was observed at

[††] These concentrations are determined using the molecular weight of Hp·2HCl, 671.6g/mol, provided by the supplier and ignoring the tendency of porphyrins to form polymeric complexes. As used here, 1mM Hp is equivalent to 0.67g/L Hp.

approximately 6-10s. All subsequent tests of compounds for sonotoxicity were performed with a 10s insonation period, since the greatest absolute difference in survival between positive and negative controls gives the most reliable estimate of sonotoxic effect.

2.8 Flow Cytometry Procedure

Stock DNA staining solution containing propidium iodide was prepared by dissolving 10mg propidium iodide in 10mL ddH_2O. A 100µg/mL stock solution of RNase A was prepared by adding 2mg of RNase A to 20mL ddH_2O. This solution was stored at 4°C and protected from light. The final staining solution was then prepared by adding 10µL of Triton X-100, 200µL of propidium iodide stock solution and 1mL of RNase A stock solution to 9mL of 1xPBS. The staining solution was stored at 4°C and protected from light. Approximately 5×10^5 cells were pelleted by centrifugation at 200×gravity for 5 minutes, and the supernatant solution was removed by vacuum aspiration. The cells were resuspended in 5mL of 1xPBS, pelleted under the same conditions, and the supernatant removed. The cells were then resuspended in 0.5mL of cold DNA staining solution using a 1000µL Eppendorf pipet. The cell suspension was stored on ice until flow cytometry analysis (Vindeløv *et al.*, 1983).

Flow cytometry was performed with a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) at the Flow Cytometry Unit, Faculty of Medicine and Oral Health Sciences, University of Alberta. Cell aggregates were gated out and

counts were plotted against fluorescence intensity. The resultant histogram was analyzed by computer to estimate the proportions of cells in each stage of the cell cycle.

2.9 Dose Fractionation

For dose fractionation experiments, HL-60 cells were concentrated to a density of approximately 5×10^6cells/mL by aspirating the supernatant medium from cells that had not been resuspended. An aliquot of 11mL of the suspension was then introduced to the insonation cuff and insonated for 10s to deplete the sonosensitive subpopulation. The cells were removed from the cuff and placed in a T-flask containing 100mL of warm medium. Insonation to generate time-dependent survival curves was carried out as described in Section 2.5.3, either immediately or after post-incubation for 30 or 90 minutes.

2.10 Numerical Methods

2.10.1 Treatment of Approximately Linear Data Sets

To analyze tests of the effect of solvent concentration on sonotoxicity, linear regression lines were computed to relate the surviving fraction of cells to the solvent concentration. Analysis of variance was used to test the significance of the relation observed between survival and solvent concentration against a null hypothesis of no relation.

2.10.2 *Survival Analysis*

The model for cell survival kinetics developed in this thesis is

$$S(s,t)=ae^{-(\alpha+\alpha 1(s,t))t}+(1-a)e^{-(\beta+\beta 1(s,t))t} \qquad (2.1)$$

where

$$\alpha_1(s,t)=\delta_1(1-e^{-(\gamma 1+\varepsilon 1t)s}) \qquad \beta_1(s,t)=\delta_2(1-e^{-(\gamma 2+\varepsilon 2t)s}) \qquad (2.2)$$

in which $S(s,t)$ is the surviving fraction of cells

t is the time of insonation

s is the sensitizer concentration

a is the proportion of sonosensitive cells

$(1-a)$ is the proportion of sonoresistant cells

$\alpha+\alpha_1(s,t)$ is the hazard function of sonosensitive cells, where α is the hazard function in the absence of sensitizer and $\alpha_1(s,t)$ is the change in the hazard function induced by sensitizer and insonation.

$\beta+\beta_1(s,t)$ is the hazard function of sonoresistant cells, where β is the hazard function in the absence of sensitizer and $\beta_1(s,t)$ is the change in the hazard function induced by sensitizer and insonation.

The model reduces the question of time dependence to the evaluation of the parameter ε for each subpopulation of HL-60 cells. Using nonlinear regression procedures, estimates of ε and its standard error, $\delta\varepsilon$, were obtained. Each ε was then tested for significant difference from zero; insignificant difference of ε from zero indicates that the hazard function reduces to a relation with no time dependence and that the sensitizer is sono*static*. A

significant difference of ε from zero indicates that the hazard function does depend on time and that the sensitizer is sono*dynamic*.

2.10.3 Survival Curve Construction

Survival curves were constructed by first examining control survival data to estimate equal intervals along the length of the fitted insonation curve, since sampling at these points will yield approximately the best parameter estimates from the curve-fitting procedure. These times were then used for the insonation of solvent-treated samples. Four samples were insonated for each insonation time. Raw survival data were determined by dividing the cell density following insonation by the cell density prior to insonation, as described in section 2.5.3.

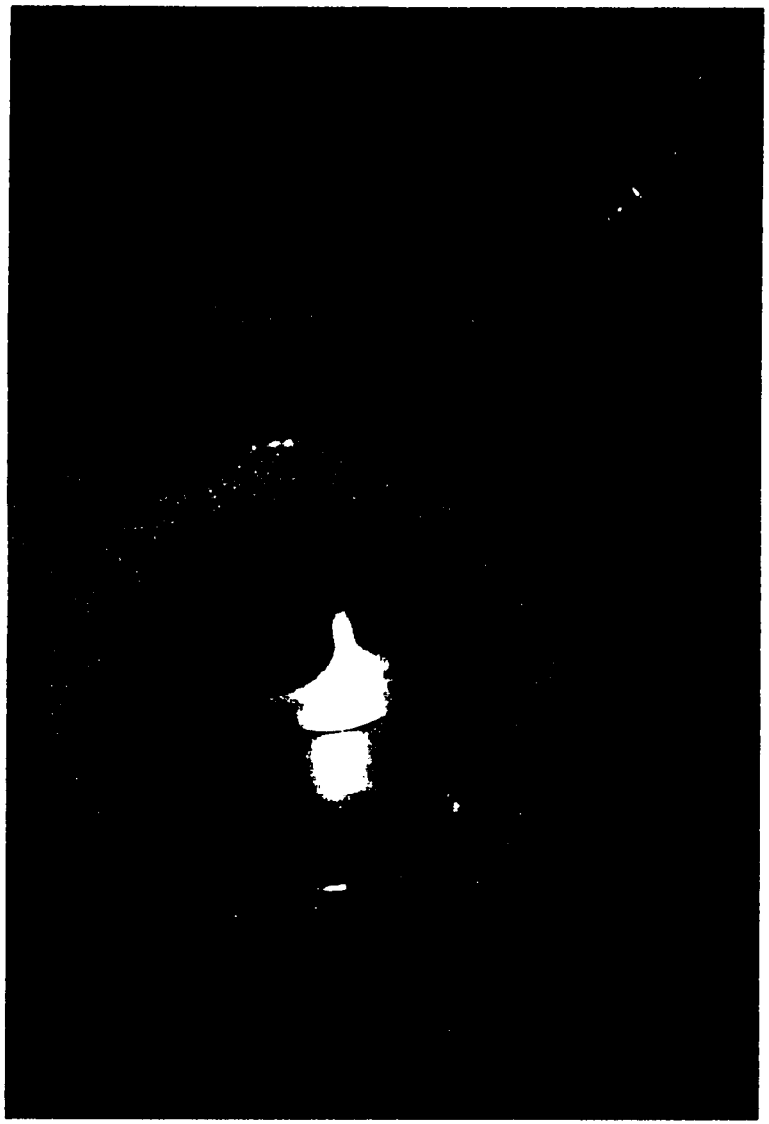
2.10.4 Statistical Analysis

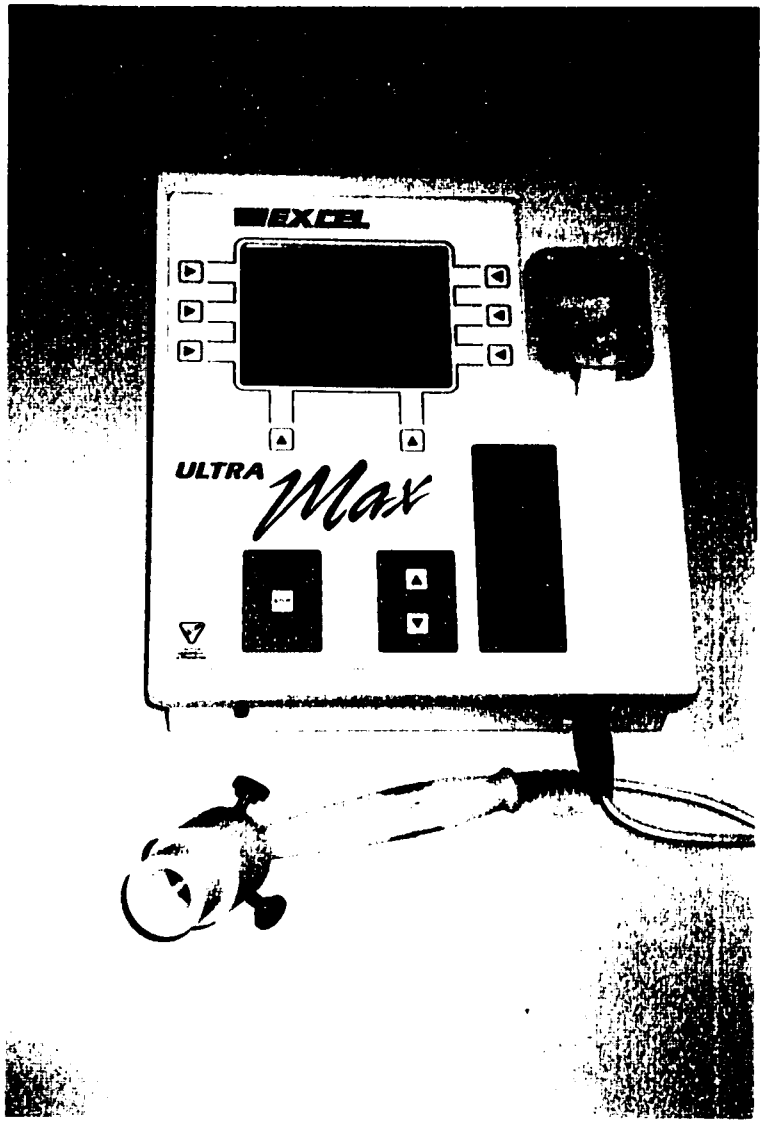
Crude initial parameter estimates were made and subsequently refined with SigmaPlot software employing the Marquardt-Levenberg (Levenberg, 1944; Marquardt, 1963; Lancaster and Salkauskas, 1986) nonlinear least squares (*nls*) algorithm. This algorithm was chosen because of its ability to progress from very poor starting values (Bard, 1970; Brown and Dennis, 1972). The refined estimates were then used as starting values for the final parameter estimation with S-Plus software using the Gauß-Newton (Gauß, as cited in Lancaster and Salkauskas) *nls* algorithm (MathSoft, Seattle, WA). This least squares estimation yielded estimates of all parameters, their standard errors, and *t*-ratios. Sample computer code and output are shown in the Appendix.

Estimates of the parameter ε were tested first for significant difference from zero using one-sided Student *t*-tests based upon the *t*-ratios calculated in the *nls* procedure. For data in which the *t*-test indicated an insignificant difference of ε from zero, extra sum-of-squares analysis (ESS) was used to evaluate the improvement in fit due to the addition of the time dependent terms (Ryan, 1997). The test-statistic was computed by dividing the residual mean-square for the reduced model (Equation 4.2.1) by the mean-square residual decrease due to the additional terms in the full model (Equations 4.2.2 and 4.3.4). This was then compared to the F-distribution cutoff for 95% confidence and the appropriate degrees of freedom (Ryan, 1997).

Plates

Plate 2.1. Top: The insonation apparatus. Bottom: The insonation cuff in enlarged view.




EXCEL
ULTRA
Max

Figures

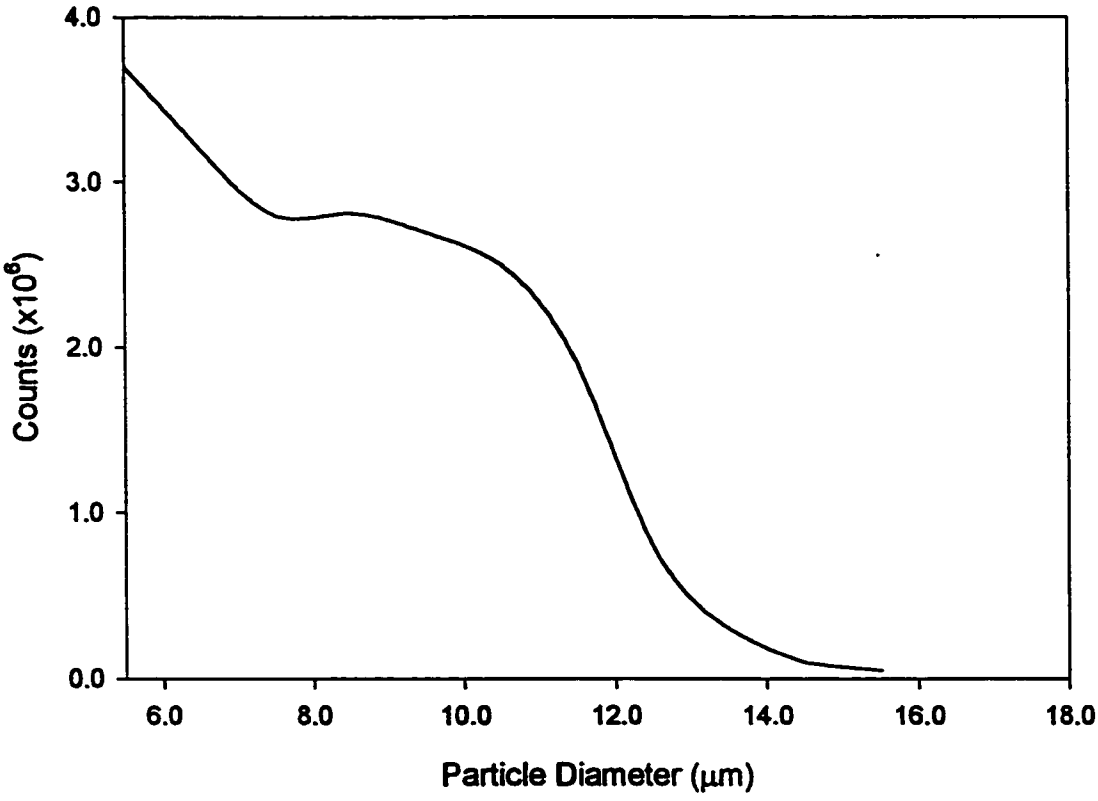


Figure 2.1. Integral count curve of HL-60 cells with respect to particle diameter. The height of the curve at each particle size corresponds to the number of particles whose diameter exceeds this size.

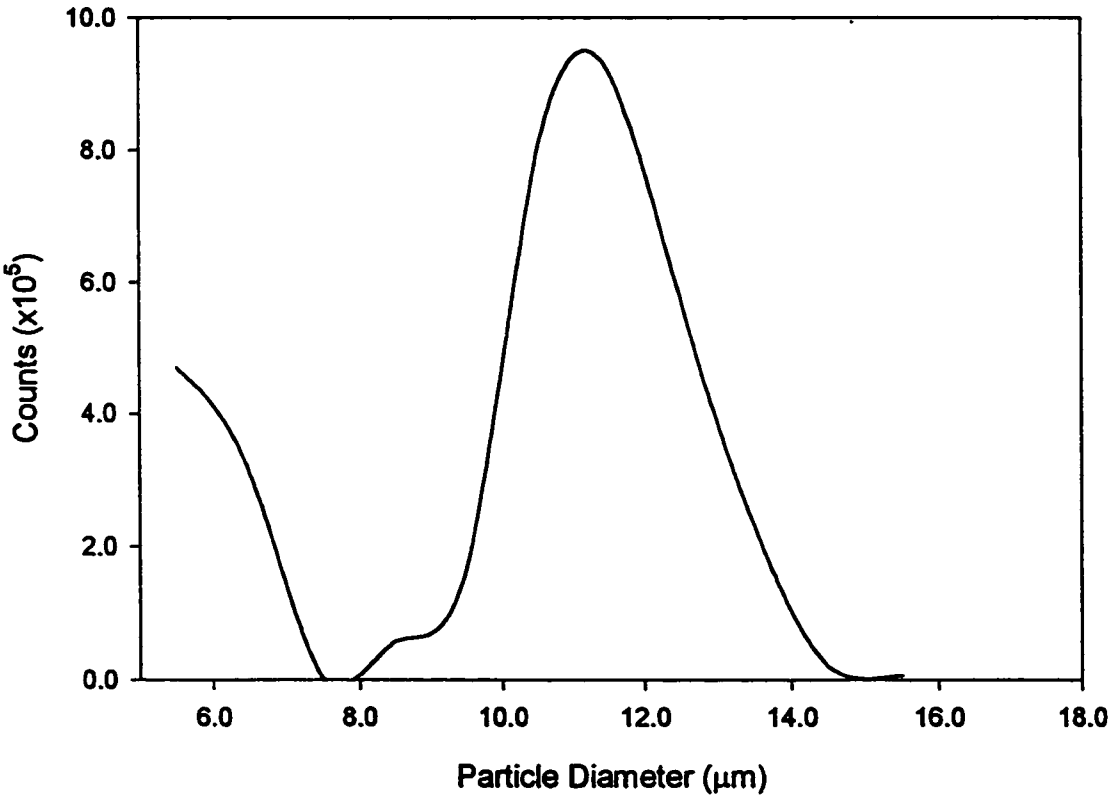


Figure 2.2. Differential count curve with respect to particle diameter showing the size distribution of HL-60 cells. Live cells range from approximately 8μm to 15μm in diameter. Cell fragments appear below 8μm. The area beneath the curve over a given interval is equal to the number of cells or fragments in that size range.

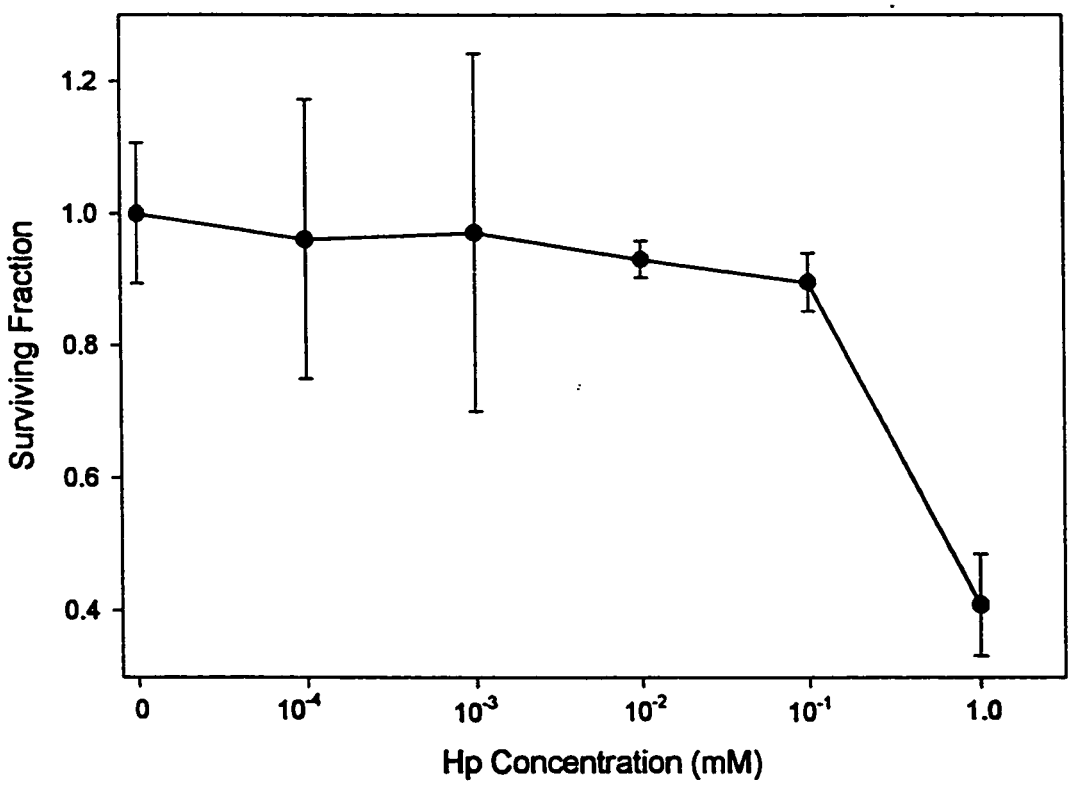


Figure 2.3. Plot of surviving fraction of HP-treated HL-60 cells versus Hp concentration for a 10s insonation. Error bars represent the 95% confidence interval of the mean at each point. No significant sonodynamic effect is observed up to 0.1mM Hp ($p > 0.1$).

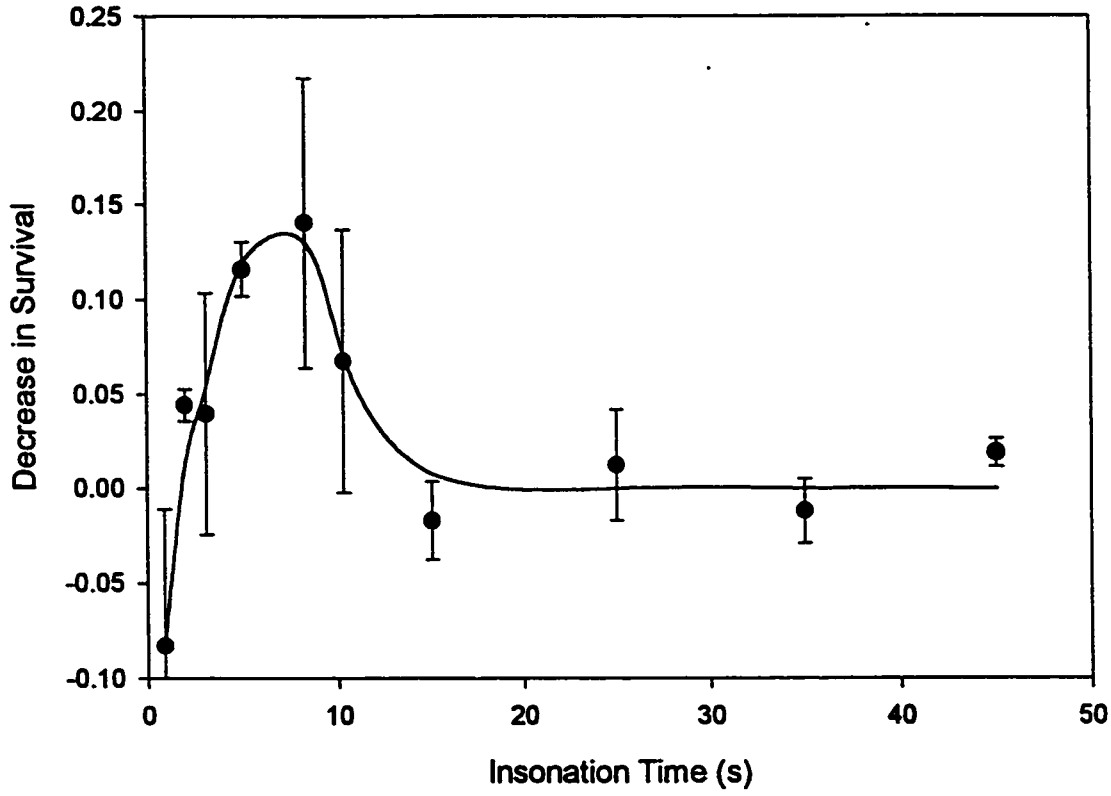


Figure 2.4. Plot of the differences between the surviving proportions of Hp-treated and control cells versus insonation time. The points are fit with a freehand curve. Error bars represent the 95% confidence interval of the mean at each point. The greatest sonodynamic effect of Hp is observed after an insonation of approximately 8s.

CHAPTER 3

Development of insonation system and cell survival assays, and tests of sonosensitizer solvent suitability

3.1 Introduction

Biological studies on sonodynamic therapy have depended on insonation apparatuses of unique design, often involving the attachment of piezoelectric transducers to radio-frequency current sources (Yumita *et al.*, 1996). Because no two devices are alike, lack of uniformity in reported results and uncertainty with respect to ultrasound dosimetry have resulted. To provide greater uniformity, a widely available and economical physiotherapy ultrasound apparatus was chosen as an instrument for *in vitro* studies. The ultrasound generator was modified by adding a customized Teflon™ cuff to the transducer to contain cell suspensions during insonation. The insonation cuff and the stainless steel surface of the ultrasound applicator are materials compatible with cell culture, and the device has a throughput of approximately thirty samples per hour for insonations of up to 30s. Further, since the apparatus was designed originally for clinical use, removal of the insonation cuff facilitates tumour control studies in animals. The apparatus is also equipped to monitor the coupling efficiency and energy transmitted to the sample.

The biological endpoints historically employed in SDT studies include assays of membrane integrity such as the trypan blue viability assay (Umemura *et al.*, 1996: *Sonodynamically Enhanced Effect of Pheophorbide A*) and clonogenic survival assays (Harrison *et al.*, 1991). The MTT assay, which exploits the ability of live cells to metabolize tetrazolium salts as an indicator of viability, could also be used to evaluate the survival of cells subjected to SDT. Such assays may be misleading since the necessary delay in counting gives surviving cells time to proliferate and may cause an overestimate of the cell density following insonation. At least one group has avoided this difficulty by using a channelyzer particle counter in assessing SDT survival, since whole cells present following

insonation are clonogenic (Kessel *et al.*, 1995). In the current work, particle size and integrity were assessed as indicators of cell death. Comparing the results to those of trypan blue assays, we conclude that since sonotoxicity is effected by physical destruction of cells, an assay based upon cell size and integrity can discern between living cells and the cell fragments produced by insonation. The assay can be performed immediately after insonation and therefore offers an estimate of the cell density immediately following the insonation period. This avoids potential misinterpretation of the degree of sonotoxicity arising from immediate regrowth of surviving cells. The advantages of the particle counter survival assay are its speed, low operating cost after the purchase of the counter, and reproducibility.

The standard method of evaluating the sonosensitizing activities of drugs has been to insonate both treated and control samples for a fixed period, comparing the observed differences in survival by the appropriate Student *t*-test. Few data have been published concerning the survival kinetics of cells exposed to ultrasound, though some survival curves have been presented to demonstrate a linear relation between *log(surviving fraction)* and insonation time (Kessel *et al.*, 1995), and a mathematical analysis of the survival of sarcoma 180 cells exposed to ultrasound has been published (Tata and Dunn, 1991). We generated survival curves for insonated, but otherwise untreated, HL-60 cells and fit an equation to these data, and found the relation identical to the model published for the sarcoma 180 cell line. In our interpretation of these results, the form of this model reveals ultrasonic killing of HL-60 and sarcoma 180 cells to be a stochastic process acting on at least two subpopulations of HL-60 cells. Tata and Dunn found that the introduction of sonosensitizer increased the rate of killing of only one subpopulation of sarcoma 180 cells.

This might indicate that subpopulations originate from partition of the cells in the insonated medium so that one subpopulation, held in place by standing waves, occupies volumes of higher ultrasonic intensity than the other subpopulation. In work presented here, flow cytometry and dose fractionation were used to further elucidate the origins of the two subpopulations.

Conventional porphyrin sonosensitizers are lipophilic, and must be dissolved in an appropriate solvent prior to administration to cell cultures. The use of low molecular weight solvents to dissolve large sensitizer molecules leaves the former in large molar excess. Since some common drug solvents have inherent sonosensitizing and differentiation-inducing properties (Jeffers *et al.*, 1995) it is important to identify compatible solvents with negligible sonosensitizing activity. Dilute sodium hydroxide has been used occasionally as a non-sonosensitizing solvent. Since NaOH is toxic, the potential sonosensitizing properties of dimethylsulfoxide (DMSO), dimethylformamide (DMF), methylformamide (MMF), and polyethyleneglycol-300 (PEG-300) were evaluated at pharmacologically relevant concentrations to assess their suitability for *in vitro*, or eventual clinical use.

3.2 Insonation System

3.2.1 Insonation Apparatus

The insonation apparatus is described in Section 2.3.

3.2.2 Ultrasonic Heating

Ultrasound-induced hyperthermia has been investigated as a tumour treatment modality for over two decades (Doss and McCabe, 1976). Ultrasound warms any medium through which it propagates, and prolonged exposure of cells to

temperatures greater than 42°C can cause cell death. To design experiments avoiding such hyperthermic effects, the rise in temperature induced by ultrasound was monitored in standard RPMI cell culture medium as a function of insonation time as described in Section 2.5.1. The results are summarized in Figure 3.1.

This experiment indicates that for insonations using this apparatus and which are begun at an initial temperature between 20°C and 22°C, it is possible to expose the cells to the highest intensity ultrasound available (2.0W/cm^2) for approximately 2.5 minutes without causing the temperature of the culture medium to exceed 40°C. Insonations begun with the medium at 37°C may be continued for approximately 30s before reaching this hyperthermic temperature range.

3.2.3 The Survival Assay

The particle-counter based survival assay system is described in Section 2.6.

3.2.4 Evaluation of the Survival Assay

In previous studies of sonodynamic cytotoxicity, the clonogenicity of cells surviving SDT was not found to be different from that of untreated control cells (Kessel *et al.*, 1995).

The effect of insonation on the number and size distribution of intact cells was examined by insonating exponentially growing suspensions of HL-60 cells for varying times at a frequency of 1MHz and an ultrasonic intensity of 2.0W/cm^2. Differential cell diameter curves were constructed using the particle counter as described in section 2.6.2.

Figure 3.2 shows the size distribution of HL-60 cells insonated for times ranging from zero to sixty seconds. The diameter of untreated control cells ranges from 9-15μm, which is consistent with a mean cell diameter of 12μm for HL-60 cells (Gallagher *et al.*, 1979). The number of living cells decreases with increasing insonation time, as indicated by the decreasing area beneath each differential curve, and a region of cell fragments appears below a particle diameter of approximately 8μm. Since no cell has a diameter greater than 15μm, setting the lower counting threshold at 9μm accounts for all living cells while excluding the cellular debris produced during insonation. Ultrasound-mediated cell disruption occurs during insonation and is complete upon cessation of insonation, as shown by the presence of whole, viable cells in Plate 3.1B.

Such size distributions permit distinction between living cells and the cell fragments remaining upon cessation of insonation, and facilitate calculation of surviving fractions based upon comparisons of the number of particles larger than 9μm in diameter before and after insonation.

3.3 Survival Characteristics of HL-60 Cells

3.3.1 Survival Analysis of Insonated HL-60 Cells

Preliminary results suggested that HL-60 cells express sonotoxic effects *in vitro* in a biphasic curve of the form noted by Tata and Dunn for sarcoma 180 cells:

$$S(t) = ae^{-\alpha t} + be^{-\beta t} \qquad (3.1)$$

where $S(t)$ is the surviving fraction of cells

t is the time of insonation

a, α, and β are constants

To accommodate a surviving fraction of 1 when t=0, the equation was refined to

$$S(t)= ae^{-\alpha t}+ (1-a)e^{-\beta t} \qquad (3.2)$$

This relation has several biological implications. Since this survival function is composed of exponential terms, it indicates that the ultrasonic killing of HL-60 cells in culture medium is stochastic and that the probability of death for any cell is independent of previous insonation. Further, since it contains two exponential terms, it indicates that the HL-60 cell population is composed of two detectable groups of cells with different sensitivities to ultrasonic destruction.

Upon these assumptions, the constants a, α, and β above may be redefined so that

a is the proportion of sonosensitive cells

α is a constant describing the sensitivity of sonosensitive cells

$(1-a)$ is the proportion of sonoresistant cells

β is a constant describing the sensitivity of sonoresistant cells

To rule out systematic deviation of the model from the observed data, the survival data were fit to the above equation and tested for lack-of-fit using statistical software (MathSoft, Seattle, WA). No significant lack of fit was found (p= 0.17) for this model. This process is summarized in Figure 3.3 and the computer program used in the procedure is reproduced in the Appendix.

3.3.2 Cell Sensitivity and Cell Cycle Stage

The effects of dose fractionation and cell cycle phase on cell sensitivity were investigated to discern the origins of the two subpopulations of HL-60 cells. Association of either of these subpopulations to a cell cycle stage or a relation of the size of either subpopulation to the incubation period between fractionated doses of ultrasound would relate origins of the two populations to cell growth. Flow cytometry (FC) analysis of the cell cycle provides an indirect observation of the relation between cell cycle stage and each subpopulation. Ultrasonic dose fractionation allows an evaluation of the ability of a depleted subpopulation to be replenished via biological processes.

The relation of cell cycle phase and sensitivity of HL-60 cells to ultrasonic destruction was investigated by FC analysis of cell nuclei stained with propidium iodide as described in Section 2.8. Table 3.1 shows the proportion of cells at each stage for uninsonated and insonated cells. No significant differences were found between the cell cycle distributions of uninsonated and insonated cells ($p>0.5$) indicating no preferential depletion of cells in any stage of the cell cycle.

Ultrasonic dose fractionation experiments were performed to study growth-related partition of HL-60 cells into sensitive and resistant subpopulations, as described in Section 2.9. The results are summarized in Figure 3.4. The increasing proportion of sonosensitive cells with increasing incubation interval between doses shows that the sonosensitive subpopulation can be regenerated from the sonoresistant subpopulation within 60-90 minutes following cessation of insonation.

The significance of the dose fractionation result is twofold. First, the subpopulations of HL-60 cells are not generated by physical assortment of the cells into the more intense and less intense field regions of a standing wave pattern. Were this so, the intense field regions depleted of cells in the preinsonation step would be immediately repopulated by cells from regions that experienced a lower field intensity. This would cause the proportion of sensitive cells to be constant with post-incubation time. Second, the increase in the proportion of sensitive cells with post-incubation time indicates that the two populations are generated by a mechanism associated with cell growth. Finally, this would suggest that the FC cell-cycle data are misleading. Ultrasonic destruction releases cell fragments, including nuclei, into the medium, so PI staining and analysis of surviving cells also stains these freed nuclei. When the cells are lysed in preparation for FC, the nuclei already freed by insonation are indistinguishable from those freed by the FC procedure. Consequently, selective depopulation of certain phases of the cell cycle cannot be shown by FC analysis.

3.4 Effects of Solvents on Cell Survival

Most sonosensitizers are lipophilic, so suitable solvents must be used for their *in vivo* administration. Since several small-molecule organic solvents demonstrate sonosensitizing activities (Jeffers *et al.*; Misik and Riesz, 1995), the relationships between sonosensitization and solvent concentration of DMSO, DMF, MMF, and PEG-300 were examined over the concentration range 0.0% to 1.0% (v/v). The results for the four solvents tested are presented in Figures 3.5 to 3.8. These experiments are necessary since the low molecular weight solvents may be present in large molar excess compared to the large sensitizer molecules they are used to dissolve.

To estimate the effects of solvent concentration on sonotoxicity, the data were subjected to linear regression analysis as described in Section 2.10.1. The data for the four solvents are summarized in Table 3.2. For all solvents tested, there was a statistically significant decrease in cell survival with increasing solvent concentration.

The effects of solvent treatment upon the proportions of sono-sensitive and -resistant HL-60 cells were also evaluated. The proportions of resistant cells in solvent-treated samples were compared to those of control samples by means of a two-sided *t*-test. The results are presented in Table 3.3. The finding of an insignificant difference in the subpopulation proportions after treatment with each solvent provides no evidence that these solvents affect the partition of cells into sonosensitive and -resistant groups. This indicates further that these are appropriate solvents for the administration of other sonosensitizers.

3.5 Discussion

The ultrasound generator selected for this work is a commercially available device— a standard, mass-produced instrument. Wider use of commercially available equipment would introduce greater uniformity to SDT studies, and would facilitate comparison of the experimental results of different research groups. The ultrasound generator is also approved for human use, which may allow its preclinical and clinical use for the testing of sonosensitizers developed with it *in vitro*.

The data assembled to study temperature increase in cell suspensions indicate that brief (<30s) insonations may be performed *in vitro* at physiological

temperatures, and that insonations of up to 2.5 minutes may be performed beginning at room temperature. Longer insonations will be possible *in vivo* due to the dissipation of the ultrasonic energy through a greater tissue mass and the further dissipation of the heat energy by flowing blood.

Cells were insonated and observed with a microscope to determine the degree of cellular integrity in the suspension following insonation. The whole cells and membrane 'ghosts' shown in Plate 3.2B were compared to cell size distributions generated from insonated cells. From these, cells were defined as particles ranging in diameter from 9μm to 15μm, and cell fragments were defined as particles whose diameter was less than 9μm. Particle count assays therefore provide a rapid means of quantifying the extent of cell death due to insonation treatment. It is also a real time assay, giving an estimate of the proportion of surviving cells immediately following insonation, and not allowing the survivors time to replicate and alter the observed counts.

Use of a mathematical model to describe this system facilitated examination of the deterministic effects of ultrasound on HL-60 cells. The model, tested for lack-of-fit to ensure that it accurately represents the survival data, showed that HL-60 cells are composed of two detectable subpopulations with different sensitivities to ultrasonic destruction. The exponential form of the model indicates that ultrasonic killing is a stochastic process for both populations. The evidence from the model showing the random nature of HL-60 cell death in an ultrasonic field, and the photographs of insonated cells showing whole cells and fragments, strongly suggest that the mechanism of cell destruction is entirely physical, though it may be affected by agents which weaken the cells to

ultrasonic tidal forces[§§] or resonance[‡]. Agreeing with the results of Kessel *et al.* (1994) from their investigation of murine leukemia 1210 cells, these data show ultrasound-induced cell death to be a stochastic process, where cell fragments represent irreversible cytotoxicity at the completion of insonation. The stochastic nature of ultrasound-induced cell death indicates that there is no accumulation of sublethal damage in insonated cells, and that all cells which survive the insonation intact are potentially clonogenic.

It should be noted that though this is the same conclusion that would be drawn based upon radiobiological principles, the processes are fundamentally different. The randomness of cell killing by ionizing radiation is a property of the incident radiation— that it strikes cells randomly. In the ultrasonic system, the randomness of cell death is a property of the cells themselves: they become unstable and have a particular probability of rupturing due to the ultrasonic stress.

Since the solubilization and administration of sonosensitizers in dilute aqueous NaOH is not pharmacologically desirable, the sonotoxic effects of four organic solvents were tested. The choice of a solvent for the administration of sonosensitizers requires an assessment of their effects at relevant concentrations in order to choose one with low background sonotoxicity and biological effects. Tests of their effects on the partition of HL-60 cells into

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[§§] Tidal forces derive from differences in the magnitude of acceleration at different points along an axis when the acceleration is parallel to that axis. The most straightforward example of a tidal force is the stretching of a free-falling parachutist whose feet are subject to a greater gravitational acceleration than his head since they are closer to the earth.

[‡] Resonance refers to the reflection and repropagation of waves so that they remain in phase where reflected waves re-enforce newly propagated waves. The destructive potential of waves resonating through a body was shown on a macroscopic scale by the wind-driven oscillations of the Tacoma Narrows Bridge, which collapsed on 7 November 1940.

sensitive and resistant subpopulations showed no significant changes. Tests of concentration effects showed that each solvent had demonstrable sonotoxicity, PEG-300 having the lowest, though none is large enough to introduce an unacceptable background effect into sonosensitizer tests.

Tables

Table 3.1. Cell Cycle Distribution of Uninsonated and Insonated HL-60 Cells.

Cycle Phase	Uninsonated (%)	Insonated. (%)	Difference (%)	*p*-value
G0-G1	49±7	48±4	0.8± 7	$p>0.5$
G2-M	10±1	10±4	-0.2± 3	$p>0.5$
S	41±8	41±7	-0.6± 9	$p>0.5$

Table 3.2. Significance of Sonosensitizing Activity of Four Drug Solvents.

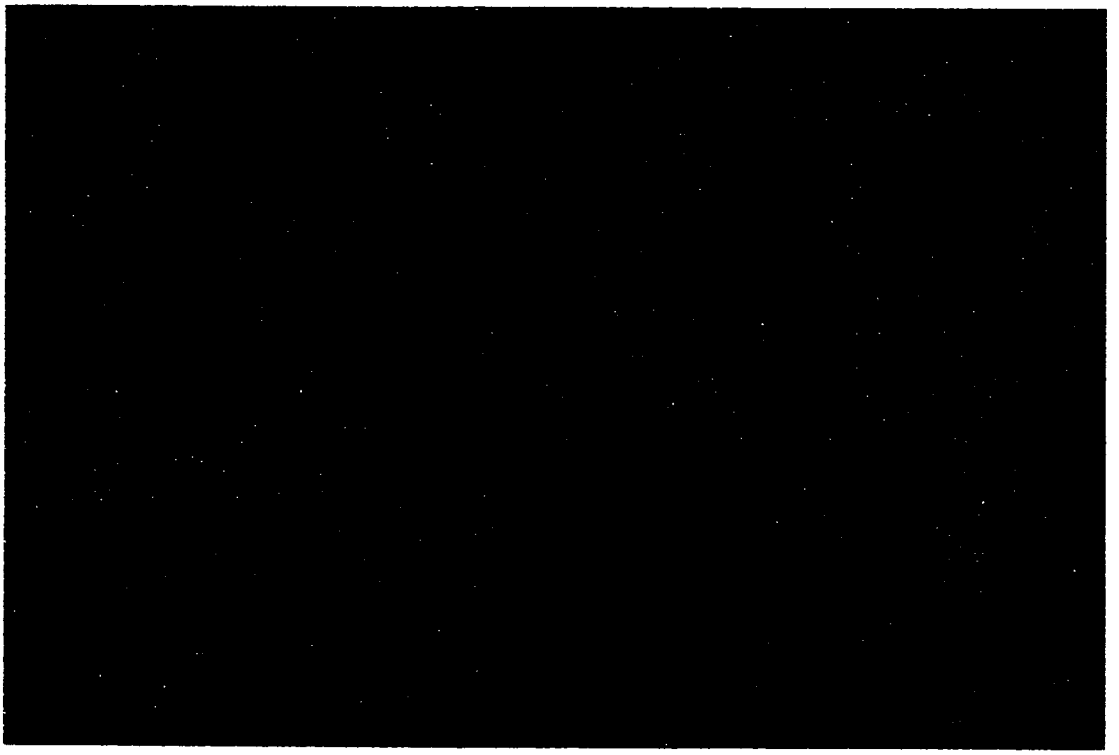
Solvent	Slope	Degrees of Freedom	Variance Ratio	*p*-value
DMSO	-0.184	70	63.23	$p<<0.01$
DMF	-0.302	70	120.89	$p<<0.01$
MMF	-0.241	70	88.34	$p<<0.01$
PEG-300	-0.055	69	6.65	$0.01<p<0.05$

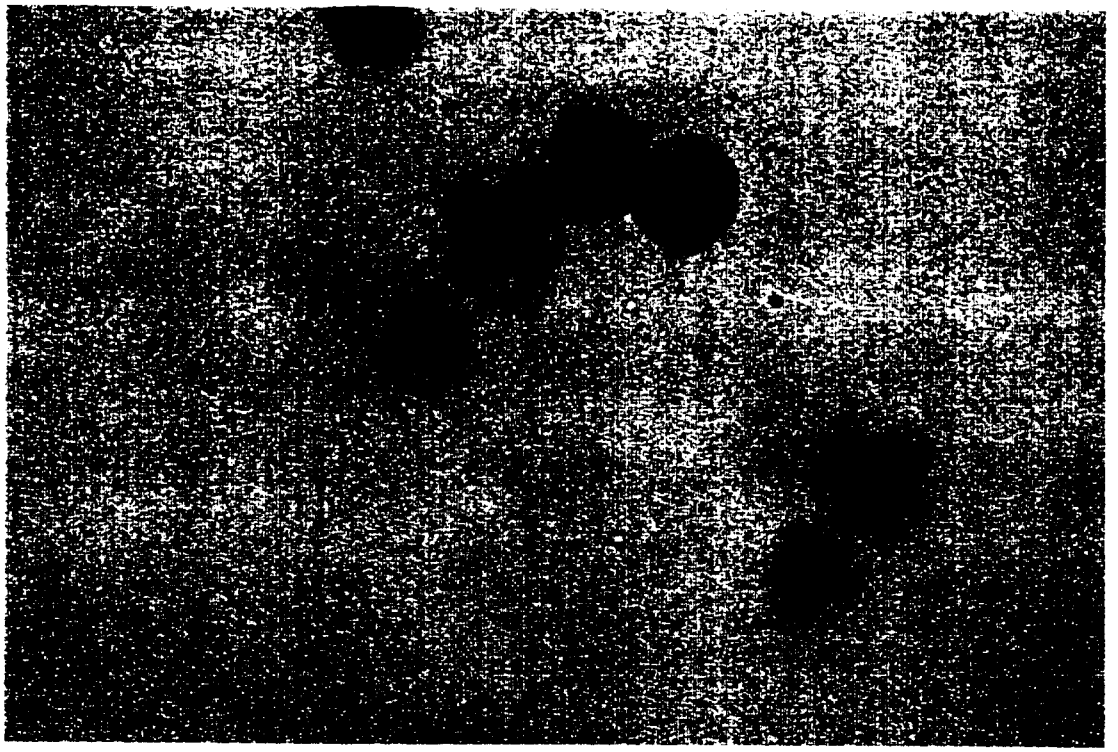
Table 3.3. Differences In Proportion of Sonoresistant Cells Between Control and Solvent-Treated Samples

Sample	Difference from Control	*p*-value
DMSO at 1%	0.034	$p>0.2$
DMF at 1%	0.11	$p>0.2$
MMF at 1%	0.055	$p>0.2$

Plates

Plate 3.1. Hematoxylin and eosin stained HL-60 cells. Top: Before insonation. Bottom: Following 10s insonation. Note the membrane ghosts among the insonated cells.





Figures

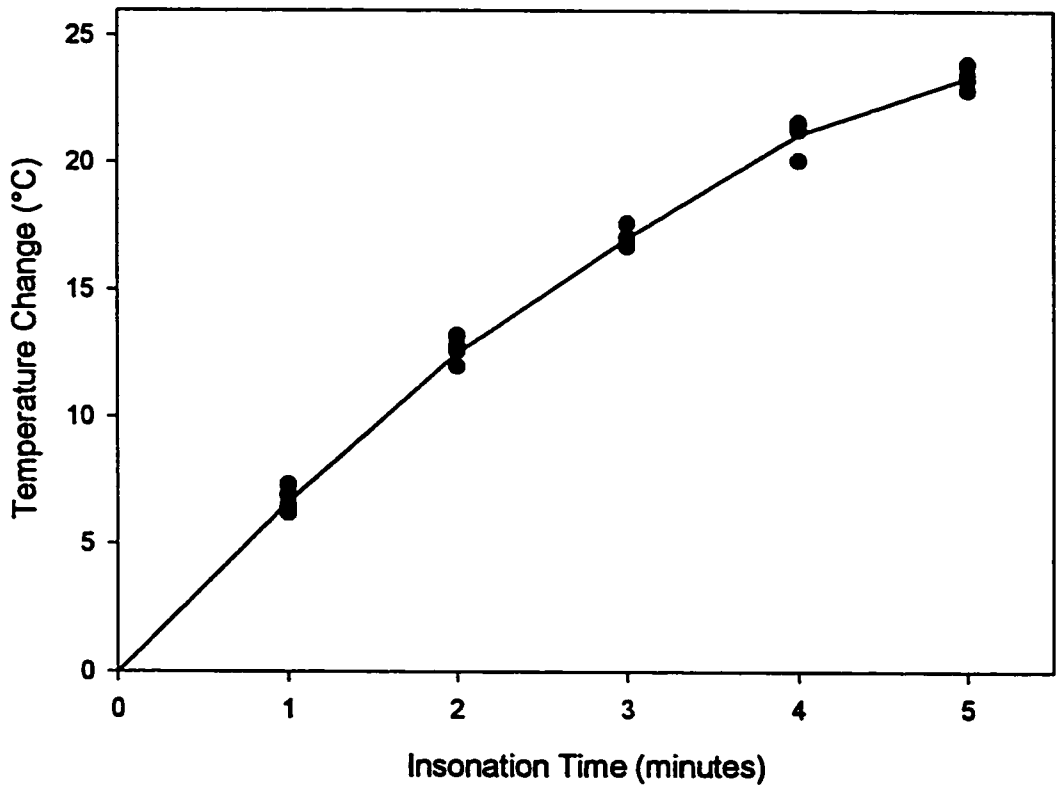


Figure 3.1. Plot of temperature change *vs.* insonation time at 2.00W/cm^2 for 10mL of standard cell culture medium.

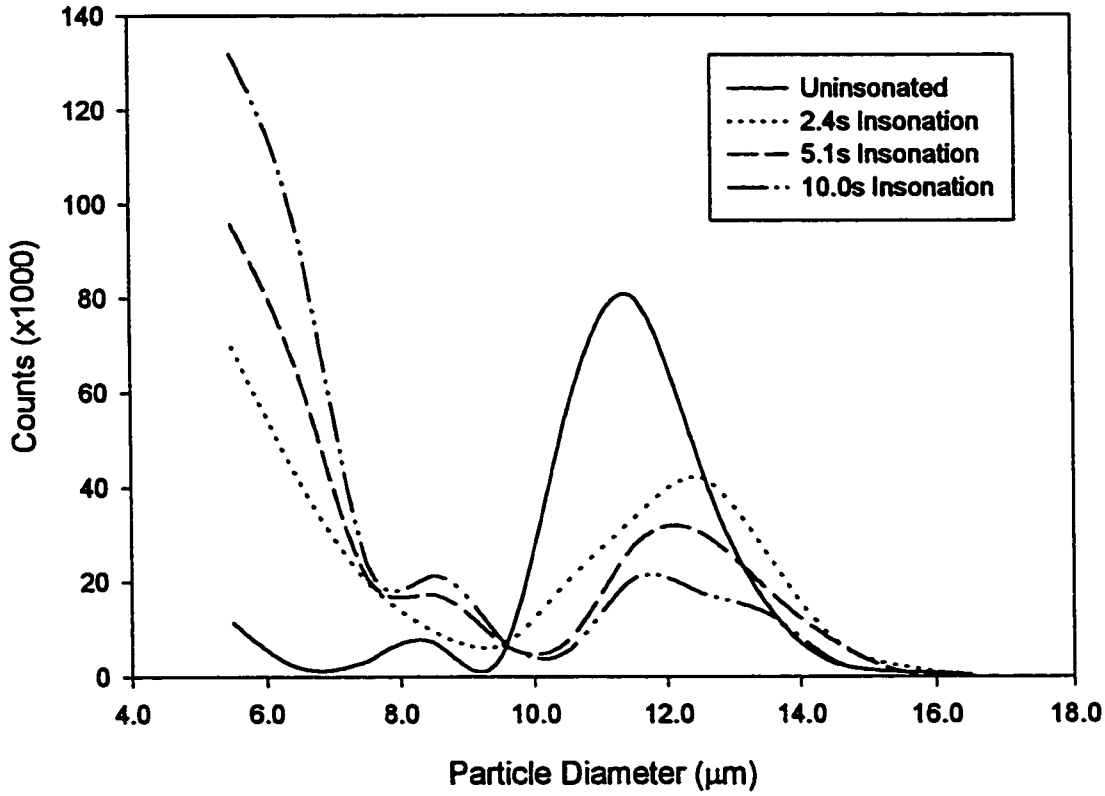


Figure 3.2. Differential particle diameter curves for HL-60 cells insonated at 2.00W/cm^2 for varying periods.

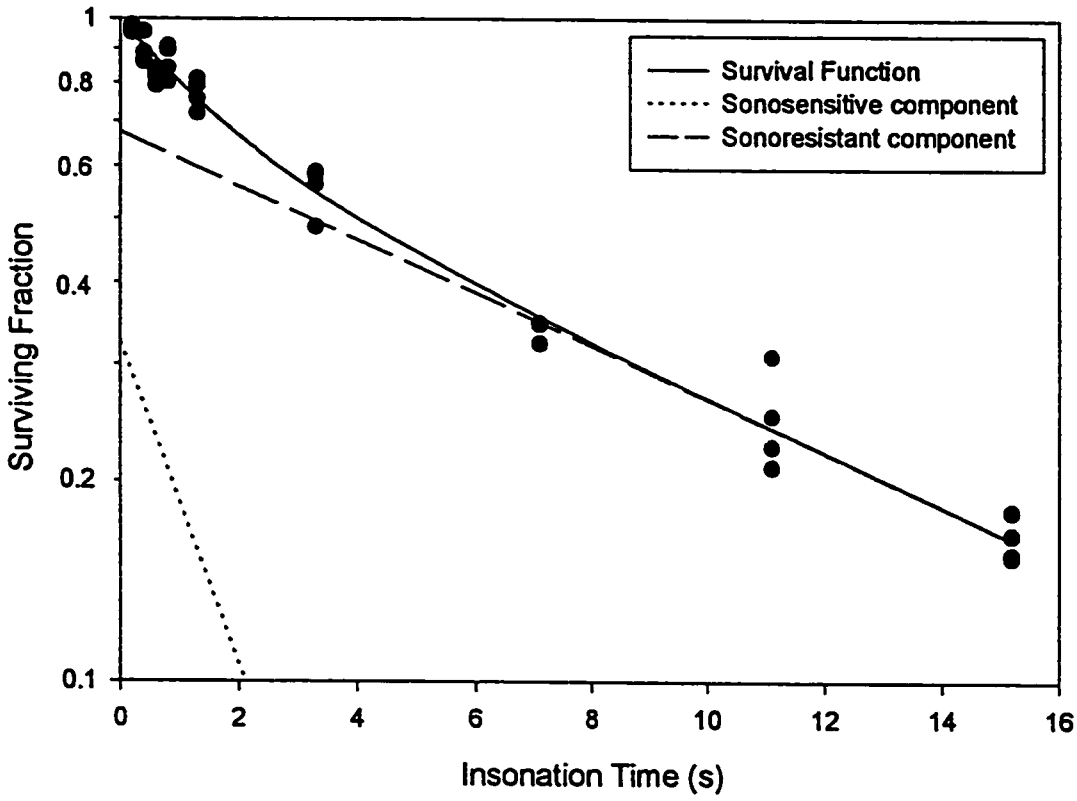


Figure 3.3. Survival curve of insonated HL-60 cells with replicate data points and fitted curve. $S(t)= 0.676e^{-0.0933t}+ 0.324e^{-0.558t}$, $r^2= 0.983$. P(Lack-of-fit)>0.2.

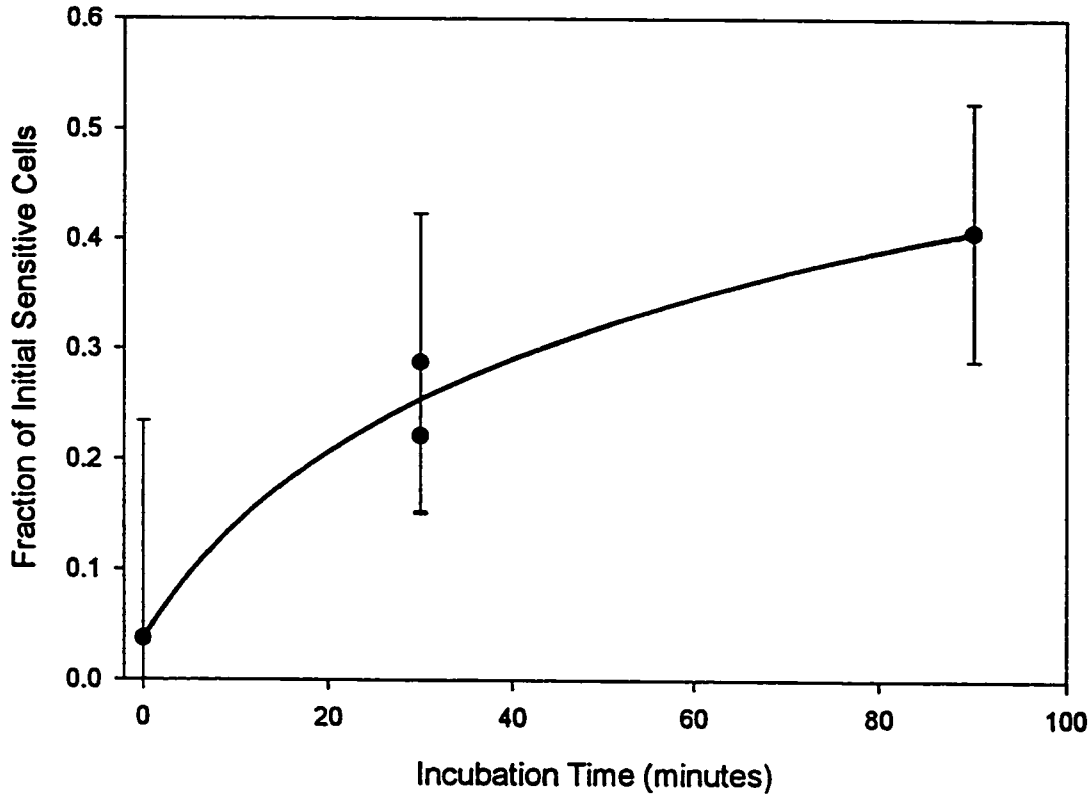


Figure 3.4. Proportion of sonosensitive cells with increasing incubation time after initial depletion.

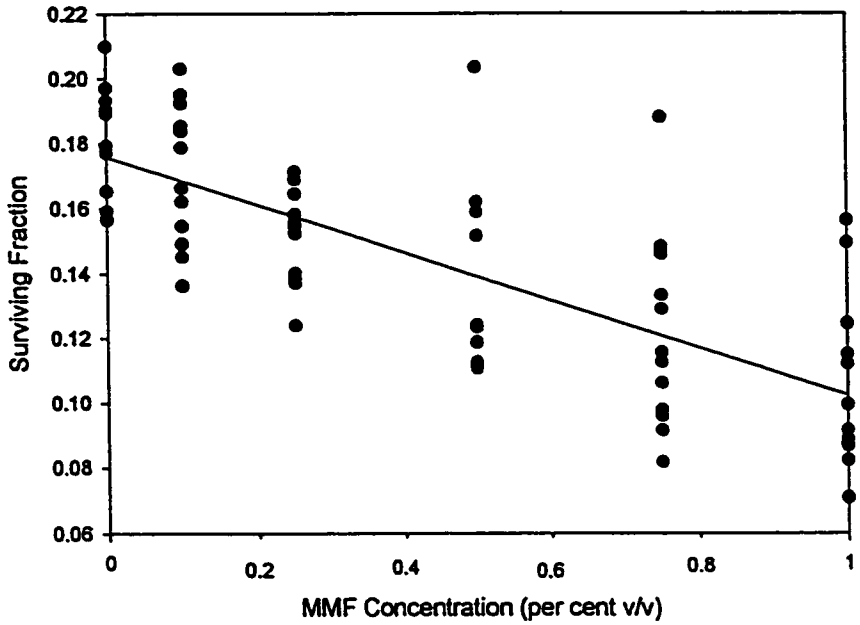


Figure 3.5. Combined data from three concentration survival curves for MMF after 10s insonations at 2.00W/cm^2. $p<<0.01$.

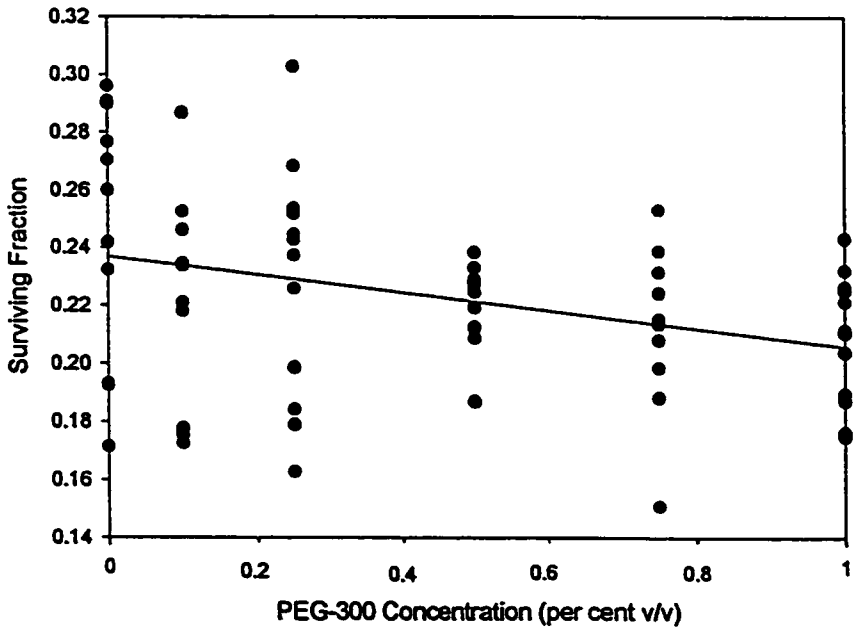


Figure 3.6. Combined data from three concentration survival curves for PEG-300 after 10s insonations at 2.00W/cm^2. $0.01<p<0.05$.

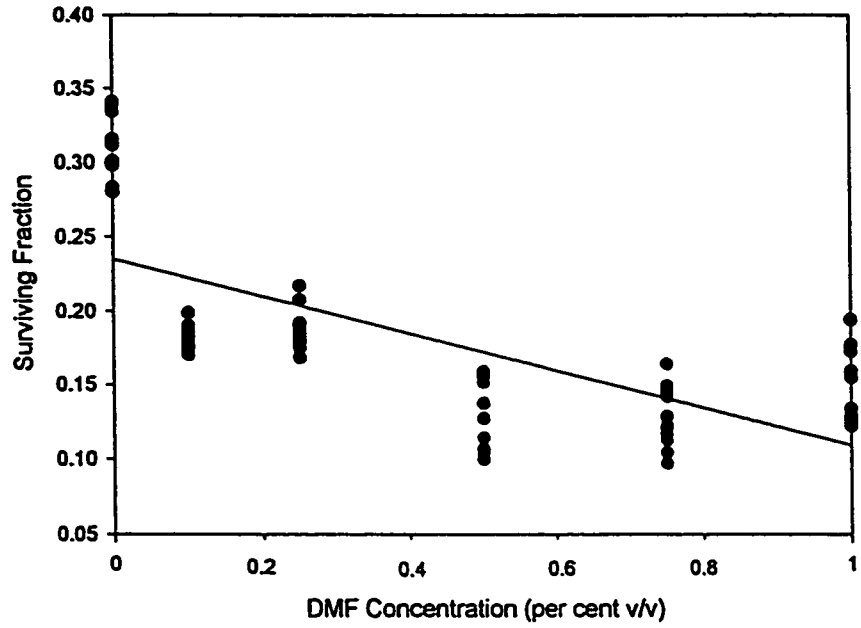


Figure 3.7. Combined data from three concentration survival curves for DMF after 10s insonations at 2.00W/cm^2. p<<0.01.

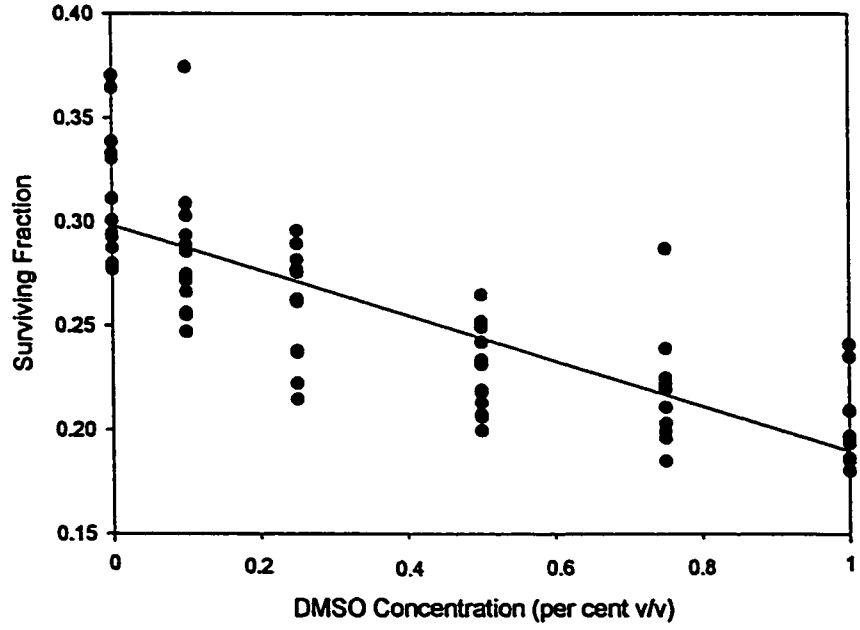


Figure 3.8. Combined data from three concentration survival curves for DMSO after 10s insonations at 2.00W/cm^2. p<<0.01.

CHAPTER 4

Statistical model for the survival of insonated HL-60 cells in the presence of membrane active sonosensitizers

4.1 Introduction

The use of photosensitizers for photodynamic therapy has been studied for about thirty years, and has yielded compounds that are clinically useful for the destruction of tumours and the palliation of advanced neoplastic disease. Useful as these compounds are, the mechanism of their action has not been demonstrated clearly. Generation of reactive oxygen and free radical species has been implicated, and results suggestive of action at the cell membrane and intracellular organelles have been reported. Sonosensitizers produce the same reactive oxygen products as photosensitizers. Though the mechanism by which sonosensitizers are stimulated to produce these products is poorly understood, it has been postulated that they are activated as photosensitizers by the sonoluminescence of cavitating water.

Survival curves are plots of the proportion of cells surviving a particular toxic treatment against a measure of the extent of treatment, such as time or energy dose. Classically, such curves have been constructed by radiobiologists to study the nature of radiation-induced DNA damage and the rate and mechanism of its repair (Puck and Marcus, 1956), and are used to study the kinetics of cell survival following any experimental treatment.

The form of the survival curve for cells subjected to ultrasound alone and ultrasound with sonosensitizer was studied by Tata and Dunn (1991), who observed first order rate kinetics for ultrasonic destruction. A system following first order kinetics is intrinsically linear, and in terms of survival analysis, this implies a hazard function[#] that is constant with respect to insonation time. A

[#] The hazard function is defined as the probability of a particular cell dying or sustaining ultimately lethal damage in any given time interval, *dt*. A hazard function related only to cell death expressed within that interval may be determined by survival assays immediately

sensitizer active at the cell membrane will weaken the cell membrane progressively as insonation continues, increasing the hazard function with respect to insonation time and producing an intrinsically *nonlinear* system. Such a sensitizer is membrane active and *sonodynamic*[§§§] insofar as added to its presence, there is an increasing secondary chemical effect potentiated by light or ultrasound. This is in contrast to two other cases. First, certain sensitizers might also localize to the cell membrane, altering the fluid mosaic and either weakening or strengthening its structure, but exerting no secondary chemical effect. Such sensitizers would increase the hazard function to a different, constant level, and may be called *membrane-active* and *sonostatic* because there is no increasing effect potentiated by light or ultrasound. In this case, the system would continue to follow first-order kinetics. Second, certain sensitizers may act within the cell, not at the cell membrane. Such sensitizers would not influence the hazard function observed in systems designed to measure the immediate destruction of cells through disruption of the cell membrane. Since damage induced by such a sensitizer occurs in the interior of the cell, it may not be expressed quickly enough to be detected by assays measuring cellular integrity immediately following insonation (Kessel *et al.*, 1995).

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following a short treatment. A hazard function related to lethal damage incurred during that interval must be determined by a clonogenic assay.

[§§§] The adjective *sonodynamic* is used here in a sense more restrictive than that originally applied to it. In the past it has been used to indicate a mechanism which increases cellular susceptibility to ultrasonic destruction without respect to the fundamental means by which this occurs. Here it is used to describe a mechanism having the effect just described, but which is mediated by the production of secondary species in quantities related to the dose of stimulating energy. This is in contrast to the term *sonostatic*, which we have coined to describe those mechanisms of sensitization which are independent of the production of toxic intermediary species in response to ultrasonic stimulation.

Several compounds have been shown to sensitize human cells to the cytotoxic effects of ultrasound. Studies of their chemical activity in cavitating aqueous medium have been performed to help explain their mechanism of action (Misik and Riesz, 1995; Umemura *et al.*, 1990). It is important to determine whether the chemical activity measured in such studies is related to the sensitization effects observed *in vitro*. Such a determination may be furthered by testing whether the toxic effects of the sensitizer vary with the time of insonation.

Survival curve analyses which can discern between first-order and alternative nonlinear systems may be useful in evaluating the mechanism of action of photosensitizers. The immediate effect of ultrasonic killing is destruction of cell membranes. The action of photosensitizers at the cell membrane (Umemura *et al.*, 1990) suggests a straightforward means of evaluating the damage to cell membranes caused by photodynamically and sonodynamically active drugs.

A model for the survival of HL-60 human promyelocytic leukemia cells in the presence of sonosensitizer and ultrasound is presented which allows the use of a simple statistical test to discern between intrinsically linear and intrinsically nonlinear kinetics. The implications of this model for the study of chemically active and inert sonosensitizers are discussed.

4.2 The Form of the Model

The first-order rate model has the form:

$$S(t)= ae^{-\alpha t}+ (1-a)e^{-\beta t} \qquad (4.2.1)$$

Where $S(t)$ is the surviving fraction of cells, t is the time of insonation, a is the proportion of sonoresistant cells, and α and β are the hazard functions of the sonoresistant and sonosensitive cells, respectively (Tata and Dunn, 1991).

The extended model has the form:

$$S(s,t)=ae^{-[\alpha+\alpha_1(s,t)]\cdot t} + (1-a)e^{-[\beta+\beta_1(s,t)]\cdot t} \qquad (4.2.2)$$

where α and β are the sensitivity constants for untreated cells as in equation 4.2.1; and $\alpha_1(s,t)$ and $\beta_1(s,t)$ are functions of solvent concentration, s, and time, t. These represent the changes in the hazard functions introduced by treatment with sensitizing compounds. The characteristic distinguishing between sonodynamic and sonostatic sensitizers is that $\alpha_1(s,t)$ and $\beta_1(s,t)$ must be functions of both s and t for a sonodynamic sensitizer. These two functions must reduce to $\alpha_1(s)$ and $\beta_1(s)$ for a sonostatic sensitizer.

4.3 Derivation of the Model

The mechanism of sensitization predicted by this model involves the mechanical weakening of the cell membrane by the localization of the sensitizing compound within it. This weakening might be viewed as the introduction of faults into a crystal, disturbing its structure. A sonostatic sensitizer would accumulate in the membrane, where its presence is sufficient

to reduce the resistance of the membrane to ultrasonic stress— the ultrasound induces no additional activity. A sonodynamic sensitizer would also accumulate in the cell membrane where at pharmacological concentrations it would have a negligible effect. Ultrasound induces such a sensitizer to produce other chemical species. Since these reactive species are produced in response to ultrasonic stimulation, their quantity is related to the dose of ultrasound. These secondary species damage the membrane, and the amount of damage induced is related to their quantity.

Therefore, for both inert and active cases, $\alpha_1(s,t)$ and $\beta_1(s,t)$ must equal zero when s= 0 because there can be no change in the hazard functions in the absence of sensitizer. Likewise, $\alpha_1(s,t)$ and $\beta_1(s,t)$ must increase as s grows larger because increasing the sensitizer concentration should increase the rate of cell killing. The terms $\alpha_1(s,t)$ and $\beta_1(s,t)$ must increase to some maximum value at large s because there will be a limit to the rate of decay that is experimentally detectable. Hence,

$$\begin{array}{ll} \alpha_1(0,t) = 0 & \beta_1(0,t) = 0 \\ \frac{\partial}{\partial s}\alpha_1(s,t) > 0 & \frac{\partial}{\partial s}\beta_1(s,t) > 0 \\ \lim_{s\to\infty}(\alpha_1(s,t))=\delta_1 & \lim_{s\to\infty}(\beta_1(s,t))=\delta_2 \end{array} \qquad (4.3.1)$$

δ_1 need not equal δ_2.

A sonostatic sensitizer must have a hazard function that is constant with time, that is,

$$\frac{\partial}{\partial t}\alpha_1(s,t) = 0 \qquad \frac{\partial}{\partial t}\beta_1(s,t) = 0 \qquad (4.3.2)$$

A sonodynamic sensitizer must have a hazard function that increases with time, as damage builds in the membranes of surviving cells, so that

$$\frac{\partial}{\partial t}\alpha_1(s,t) > 0 \qquad \frac{\partial}{\partial t}\beta_1(s,t) > 0 \qquad (4.3.3)$$

The distinction between sonodynamic and sonostatic sensitizers can then be made by testing the hazard functions for time dependence. A model for the change in the hazard functions that fits the criteria outlined above is

$$\alpha_1(s,t) = \delta_1(1 - e^{-(\gamma_1 + \varepsilon_1 t)s}) \qquad \beta_1(s,t) = \delta_2(1 - e^{-(\gamma_2 + \varepsilon_2 t)s}) \qquad (4.3.4)$$

In this model, the values of $\partial/\partial t[\alpha_1(s,t)]$ and $\partial/\partial t[\beta_1(s,t)]$ are related to the values of ε_1 and ε_2. If ε_1 or ε_2 is found to equal zero, then the values of these derivatives are zero and the sensitizing compound must be sonostatic. If ε_1 and ε_2 are found to be greater than zero, then the values of these derivatives are positive and the sensitizing compound is sonodynamic.

Experimentally, determination of membrane associated chemical activity depends upon one-sided *t*-test analysis of the parameter and standard error estimates for ε_1 and ε_2 provided by nonlinear regression procedures, and extra sum-of-squares analysis of the significance of the time-dependent terms.

Significant difference of either ε_1 or ε_2 from zero indicates time-dependent activation of the sensitizer upon the associated subpopulation, which is consistent with a sonodynamic mechanism of action.

4.4 Discussion

Ultrasonic destruction of otherwise untreated HL-60 cells is a stochastic process, whereby any surviving cell has a constant likelihood of being destroyed in any differential time interval, independent of previous insonation. Time dependence of the hazard functions in this model discerns between the two mechanisms of sensitizer action outlined above. A sonostatic sensitizer, which functions by partitioning into the cell membrane of target cells and weakening them to mechanical destruction by its simple presence, will not increase cells' sensitivity to ultrasonic destruction as insonation progresses. Such cells will have a constant susceptibility to ultrasonic destruction and therefore a hazard function that is constant with (and therefore independent of) time. In the case of sonodynamic sensitizers, which are activated by ultrasound to produce reactive intermediate species, the damage to the cell membrane is related to the quantity of reactive intermediates formed. This, in turn, is related to the duration and intensity of insonation. In such a case, damage to the membrane builds as insonation proceeds, increasing the susceptibility of each cell to ultrasonic destruction. This increasing susceptibility with time requires that the hazard function increase with time. For a sonodynamic sensitizer, the hazard function must therefore be time dependent.

Evidence that both photosensitizers and sonosensitizers have membrane-associated activity (Yumita *et al.*, 1994) may allow extension of the model

presented here to photosensitizer analysis. In experimental sonodynamic therapy, ultrasonic energy not only stimulates chemical damage to cells, it creates a hostile physical environment which causes immediate expression of this damage. Experimental photodynamic therapy induces similar damage to cells, but does not result in cell death until some damage threshold has been reached. Further, it does not change the environment so that damage below this threshold can be observed directly. Standard cytotoxicity assays measure only cell death due to damage exceeding this threshold, and the shoulder on survival curves constructed for photosensitizers gives only an indirect measure of the rate of accumulation of damage to the membrane.

It may be useful, then, to note how sonodynamic conditions might be mimicked by the treatment of photosensitized cells simultaneously with light and low intensity ultrasound. Here, photodynamic activity replaces the chemical effects of sonodynamic activity, producing reactive species which attack cells. The vibrational and tidal stresses associated with insonation are provided by the low intensity ultrasound (which does not produce the cavitation necessary for sonodynamic action). This provides a system in which photodynamic damage is induced and developed simultaneously, without competition between sonodynamic and photodynamic processes. In such a system, the increased susceptibility to ultrasonic destruction induced by even low levels of photodamage may be observed more directly through the analysis of survival as a function of time according to the model presented here.

CHAPTER 5

Statistical analysis of the sonosensitizing mechanisms of three solvents

5.1 Introduction

The finding that several small polar organic solvents possess sonosensitizing activity (Jeffers *et al.*, 1995) makes it important to determine the nature of their sensitizing mechanism. This is important both to understand these substances as sensitizers in their own right, and to understand how their sensitization properties might confound studies of other sonosensitizers which require them as solvents for administration *in vitro* and *in vivo*.

The model developed in Chapter 4 for the survival of HL-60 cells insonated for various times with 1MHz ultrasound and treated with sonosensitizer at varying concentrations was used to examine the mechanisms of action of three sonosensitizing solvents.

5.2 Cell Preparation and Solvent Administration

The HL-60 cells employed in these studies were cultured, prepared and insonated as described in Sections 2.4 and 2.5. Solvents used in this experiment were dimethylsulphoxide (DMSO), *N*,*N*-dimethylformamide (DMF), *N*-methylformamide (MMF), and polyethyleneglycol-300 (PEG-300).

5.3 Analysis

Survival curves were prepared and analyzed as described in Sections 2.10.3 and 2.10.4 according to the model described by equations 4.2.2 and 4.3.4.

5.4 Results

Results from these experiments are summarized in Table 5.1. The large *p*-values for extra sum-of-squares analysis (P(ESS)) obtained for both the α and β terms indicate that there was no significant time dependence of survival

kinetics induced by any solvent sensitizer tested. The insignificance of time-dependent terms is consistent with the conclusion that DMF, MMF, DMSO and PEG-300 are sonostatic sonosensitizers whose mechanism of action does not involve the production of intermediate chemical species upon ultrasonic exposure.

5.5 Discussion

The tests described, based upon equations 4.3.1 to 4.3.4, are designed to discern between two systems, sonostatic sensitization which is intrinsically linear[†††], and sonodynamic sensitization which is intrinsically nonlinear. The data from this experiment indicate that the solvent sonosensitizers examined do not induce detectable nonlinear behaviour of the HL-60 survival function and that according to the model presented in the previous chapter, these solvents have no sono*dynamic* activity.

The most useful aspect of these results is the knowledge that treatment with these solvents, though they do increase the sensitivity of HL-60 cells to ultrasonic destruction, does not alter the kinetics of cell survival to a detectable extent. A system treated with these solvents exhibits the same intrinsically linear kinetics as an untreated system. Because of this, other sensitizers which may exhibit more complex effects can be administered to cells using these solvents without fundamentally altering the background process of the experimental system.

[†††] *Intrinsically linear* indicates any relationship between two variables that is either first-order (of the form $y = mx + b$) or can be transformed into a first-order relationship between the transformed variables. An example is the exponential decrease function, $y = e^{-x}$, which using logarithms can be transformed into $\ln y = -x$. *Intrinsically nonlinear* indicates a relationship between two variables which cannot be transformed into a first-order relationship.

Tables

Table 5.1. Significance of Nonlinearity Observed In Survival Functions of HL-60 Cells Treated with Sonosensitizing Solvents

Solvent	$P(\varepsilon_\alpha>0)$	$P(\varepsilon_\beta>0)$	P(ESS)α	P(ESS)β
DMF	>0.1	>0.1	>0.1	>0.1
DMSO	>0.1	>0.1	>0.1	>0.1
MMF	>0.1	>0.1	>0.1	>0.1
PEG-300	>0.1	>0.1	>0.1	>0.1

Chapter 6

Screening Evaluation of Novel Sonosensitizers

6.1 Introduction

The most thoroughly investigated sonosensitizing compound is hematoporphyrin derivative (HpD) which has been modified to produce Photofrin™, the only drug licensed for use in photodynamic therapy. Though effective in the photodynamic therapy of superficial tumours, these drugs have been found to cause acute cutaneous photosensitivity (Marcus, 1992; Estey *et al.*, 1996) for a period of several weeks following treatment. A new generation of photosensitizers has been developed which are both more effective than HpD and its derivatives and which result in cutaneous photosensitivity for significantly shorter periods following treatment (Reszka *et al.*, 1992; Estey *et al.*, 1996). The mechanism of action of photosensitizers has been related to their production of reactive oxygen species upon stimulation by light of an appropriate wavelength (Marcus, 1992). This has also been investigated as a possible mechanism of action of sonosensitizing compounds following stimulation by ultrasonic energy (Umemura *et al.*, 1990; Yumita *et al.*, 1994). Because of the historic and conceptual relations between photosensitization and sonosensitization, panels of quinonoid and diaminoanthraquinone (DAAQ) pigments were screened for use as sonosensitizers. These compounds have been observed to have favourable photodynamic properties and side effects that are much less severe than conventional sensitizers.

The most common diaminoanthraquinone is the hair dye 1,4 diaminoanthraquinone, which has been shown to have mutagenic properties

in bacteriophage (Kvelland, 1983). Some DAAQ derivatives have been shown to have antitumour properties (Lown, 1993) and for at least some of these, this activity is related to inhibition of protein kinase C (Jiang *et al.*, 1992). Other DAAQ derivatives, designed to minimize dark toxicity, have strong light absorption in the phototherapeutic window of 600nm to 1000nm wavelength. These have been shown to sensitize cultured K562 human chronic myeloid leukemic cells to light (Hartley *et al.*, 1990, Reszka *et al.*, 1992).

The perylenequinonoid pigments (PQP's) hypocrellin A and hypocrellin B are isolated from the fungi *Hypocrella bambuase* and *Shirala bambusicola* and possess strong photosensitizing properties (Diwu and Lown, 1990). They demonstrate high quantum yields of singlet oxygen and result in minimal post-treatment photosensitivity (Estey *et al.*, 1996). These properties have led to their derivitization and the investigation of both the parent compounds and their derivatives as potential agents for use in photodynamic cancer therapy. Certain hemiquinone compounds, related to PQP's, were included in this screen.

Six of the forty-one compounds examined, RH-700, A-6-II, $CPMg(Ac)_2$, DBHB, DMHB, and $HBMg(Ac)_2$, showed sonosensitizing activity great enough to warrant further investigation, particularly of their ultrasound-induced singlet oxygen production by EPR spectroscopy.

6.2 Methods

HL-60 cells were treated with potential sensitizers and insonated as described in Sections 2.5.2 and 2.5.3, and the surviving fractions were plotted against sensitizer concentration. This procedure allowed efficient screening of the compounds and eliminated the need for additional examination of biologically inactive compounds.

6.3 Results

Figures 6.1 to 6.39 show cell survival as a function of sensitizer concentration for all compounds tested. Error bars at each point represent the 95% confidence interval for the mean survival at that point. The average survival of the Hp positive controls is indicated in the lower right of each diagram; the error bars represent the 95% confidence interval of the mean. Among the DAAQ compounds, only one candidate, A-6-II, showed sonotoxic effect comparable to the HP control (Figure 6.2). Its maximum sonotoxic effect was approximately one-third less than that observed for the Hp positive controls.

Among the hemiquinone compounds, only RH-700 produced sonotoxic effect comparable to that of the Hp control (Figure 6.17). The survival curve showed a significant decrease in cell survival upon exposure to 0.1μM RH-700 and a slower decline in survival up to the highest concentration of 1000μM. The aberrant data point at 10μM is possibly due to inadequate resuspension of the

cells before counting prior to insonation, and the dispersion of surviving cells during insonation. This could have yielded a sample of low cell density prior to insonation, and a sample with the proper density following insonation, and therefore an overestimate of the fraction of cells surviving this treatment.

The PQP compounds yielded four candidates for further analysis. At a concentration of approximately 30μM, $CPMg(Ac)_2$ showed sonotoxicity exceeding that of the 1000μM Hp control, with the decrease in survival occurring steeply over the preceding two decades of sensitizer concentration (Figure 6.21). DBHB and DMHB showed negligible sonotoxicity up to 100μM. The bulk of the observed sonotoxic effect occurred over the decade from 100μM to 1000μM, and the maximum effects were comparable to that of the Hp control (Figures 6.22 and 6.23). $HBMg(Ac)_2$ showed no sonotoxic effect until 6μM. Cell survival decreased steeply over the next two decades of sensitizer concentration (Figure 6.38).

6.4 Discussion

The experimental design of the dilution series resulted in the systematic exposure of one tenth of each cell population to a sensitizer concentration ten times higher than the intended concentration. Though the 4 hour incubation may have allowed diffusion of the sensitizer out of the cells which had received the higher dose, so that by the end all were in contact with sensitizer at the same concentration, it is not possible to ascertain the precise effects. The

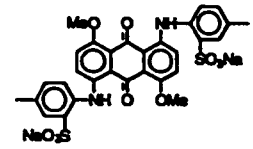
effect should only have been observed at low concentrations for those sensitizers which otherwise would produce negligible sonotoxic effect at low concentration. Because of this, it is reasonable to assume that for sonosensitizers that showed no sonotoxic effect at low doses, this systematic error in the design caused no significant complications. The observed sonotoxic effect of those sensitizers which showed an increasing effect over a wider range of concentrations may at most have been overestimated by 0.1. This maximum error would only occur if the next higher concentration caused complete cell killing, and corresponds to the ten *per cent.* of the cells in each sample that were exposed to the next higher dose.

This screen reveals six novel sonosensitizing compounds whose sonotoxic activity approximates that of Hp. Four of these compounds showed sonotoxic effects over concentration ranges that are either excessively narrow (one decade) or excessively broad (four decades). This may prevent clinical use of these compounds since a narrow range prevents easy dose adjustment to achieve the desired effect, due to the small difference between doses that would cause negligible effect or great effect. Additionally, for those compounds with sono- and photo-dynamic properties, broad concentration ranges may cause excessive residual photosensitivity after treatment. This is so because the drug may cause photosensitivity at much lower concentrations than are required for successful sonodynamic therapy, and may remain above the threshold concentration needed to cause photosensitivity for several days

following treatment. The two PQP compounds CPMg(Ac_2) and HBMg(Ac_2), however, produced significant sonotoxicity over the two final decades of concentration and insignificant toxicity below these concentrations. These two compounds may therefore be the most promising for further development as sonosensitizing compounds for the treatment of neoplastic disease.

Figures

I. DAAQ Compounds



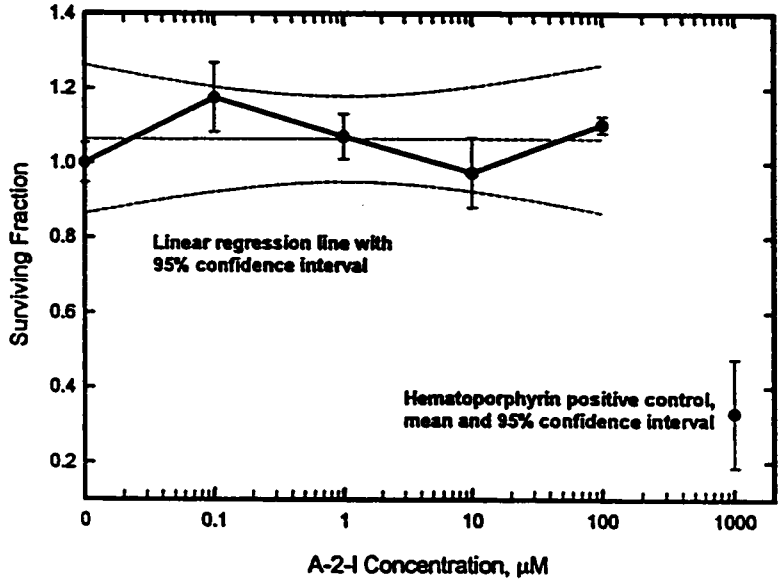
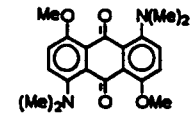


Figure 6.1 Sonodynamic activity of A-2-I in HL-60 cells given 10s insonation at 2.0W/cm^2.



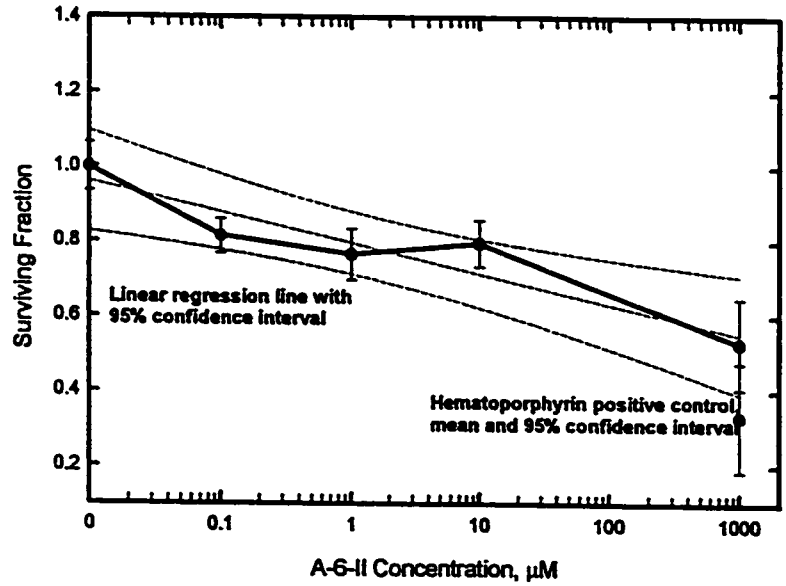
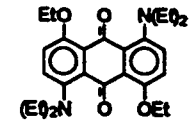


Figure 6.2 Sonodynamic activity of A-6-II in HL-60 cells given 10s insonation at 2.0W/cm^2.



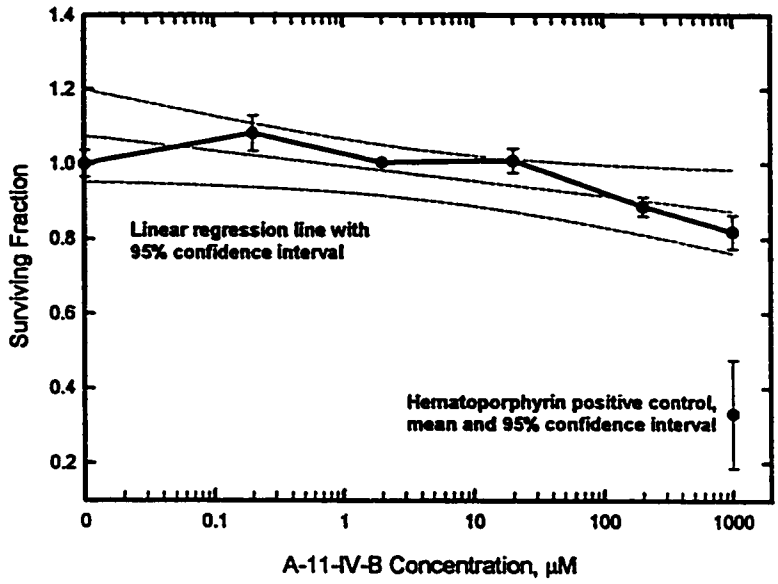
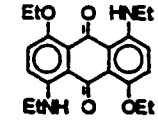


Figure 6.3 Sonodynamic activity of A-11-IV-B in HL-60 cells given 10s insonation at 2.0W/cm^2.



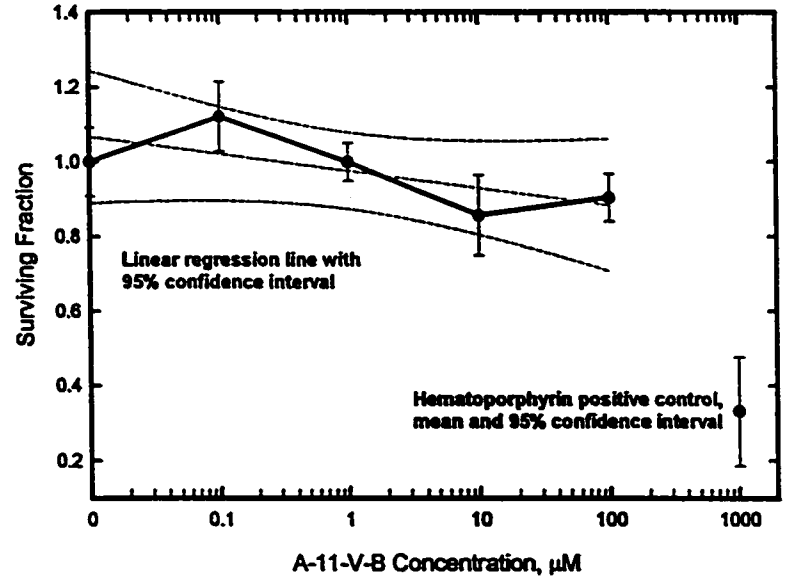
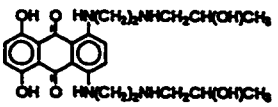


Figure 6.4 Sonodynamic activity of A-11-V-B in HL-60 cells given 10s insonation at 2.0W/cm^2.



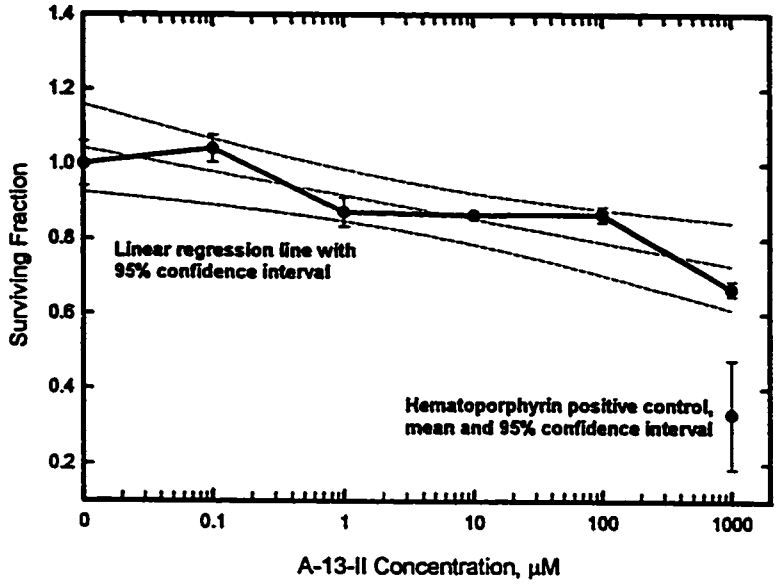
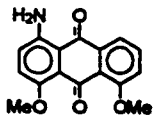


Figure 6.5 Sonodynamic activity of A-13-II in HL-60 cells given 10s insonation at 2.0W/cm^2.



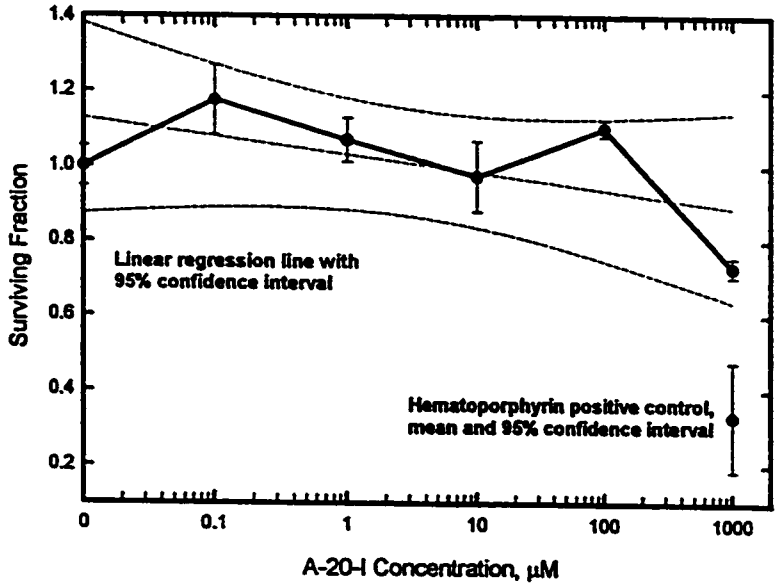
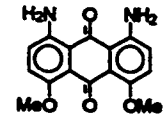


Figure 6.6 Sonodynamic activity of A-20-I in HL-60 cells given 10s insonation at 2.0W/cm^2.



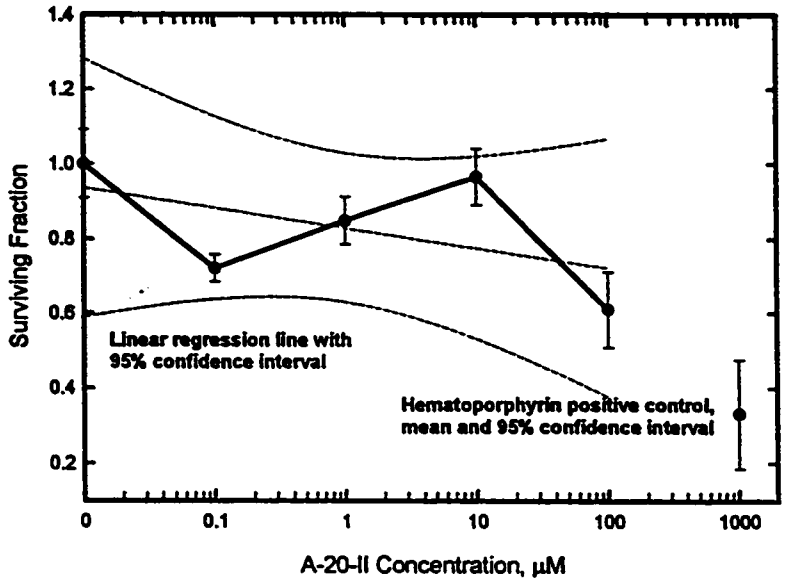
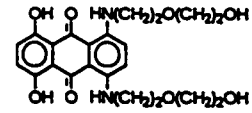


Figure 6.7 Sonodynamic activity of A-20-II in HL-60 cells given 10s insonation at 2.0W/cm^2.



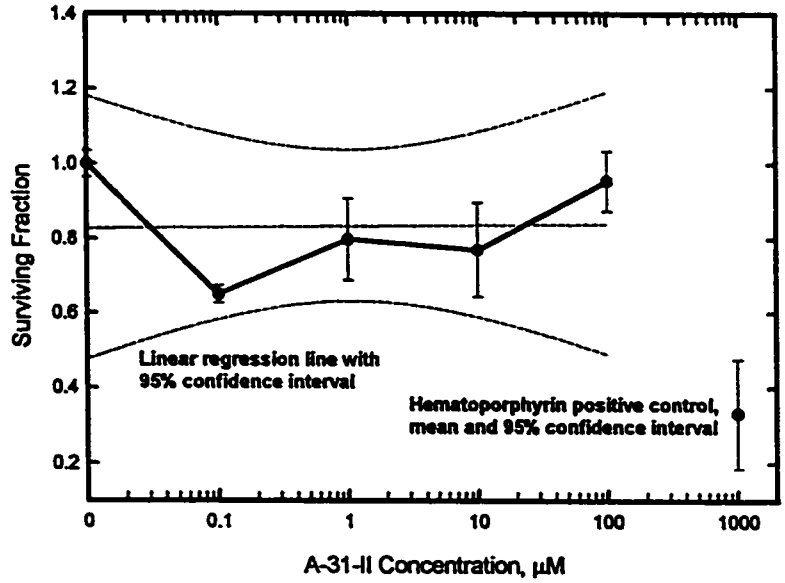


Figure 6.8 Sonodynamic activity of A-31-II in HL-60 cells given 10s insonation at 2.0W/cm^2.

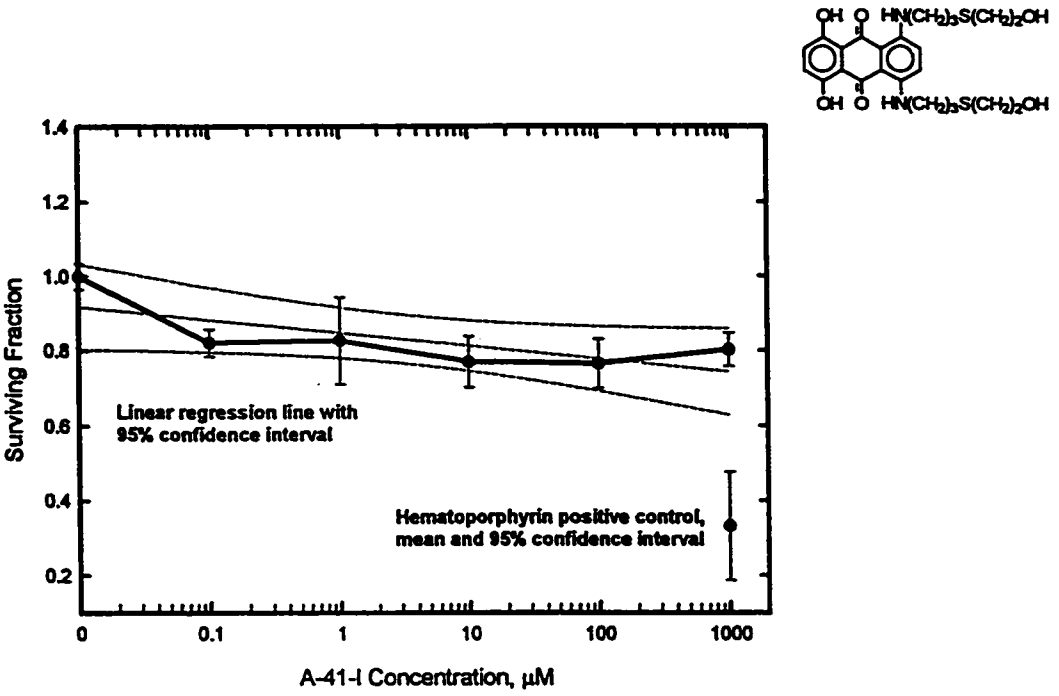


Figure 6.9. Sonodynamic activity of A-41-I in HL-60 cells given 10s insonation at 2.0W/cm^2.

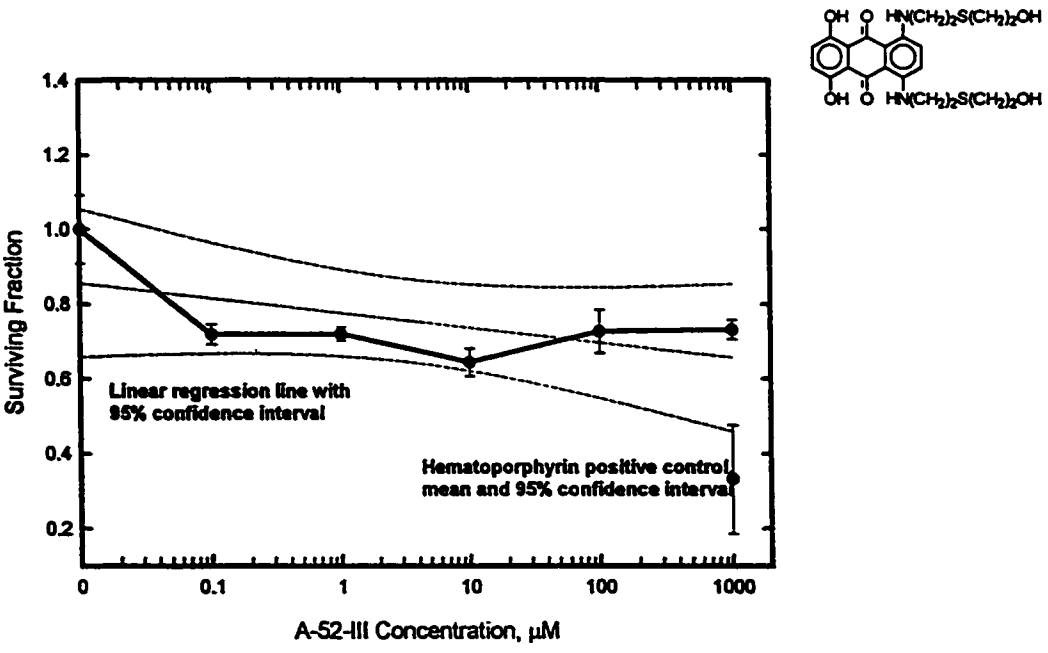


Figure 6.10. Sonodynamic activity of A-52-III in HL-60 cells given 10s insonation at 2.0W/cm^2.

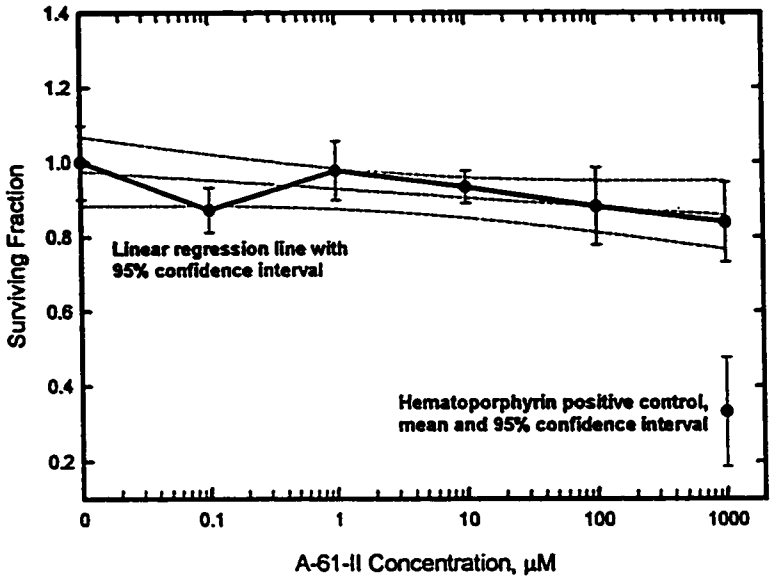
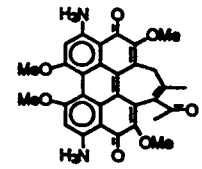


Figure 6.11. Sonodynamic activity of A-61-II in HL-60 cells given 10s insonation at 2.0W/cm^2.

II. Hemiquinone Compounds



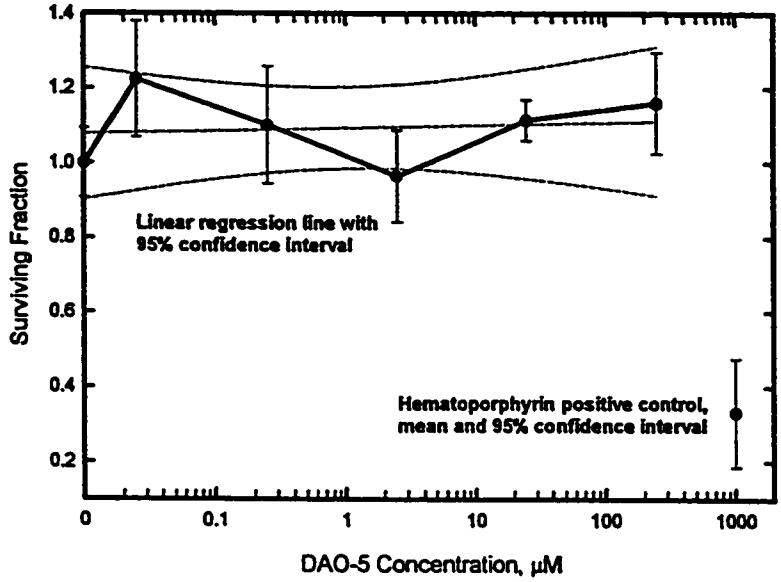
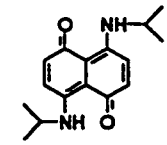


Figure 6.12 Sonodynamic activity of DAO-5 in HL-60 cells given 10s insonation at 2.0W/cm^2.



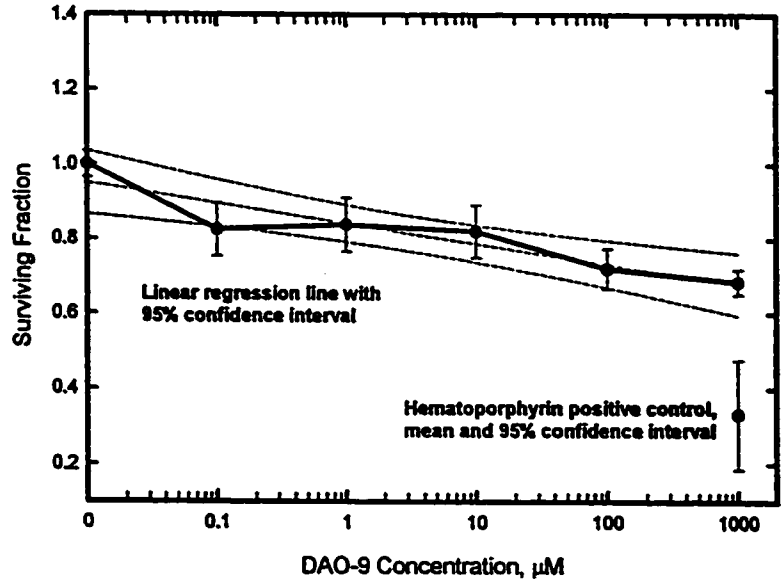
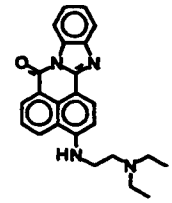


Figure 6.13. Sonodynamic activity of DAO-9 in HL-60 cells given 10s insonation at 2.0W/cm^2.



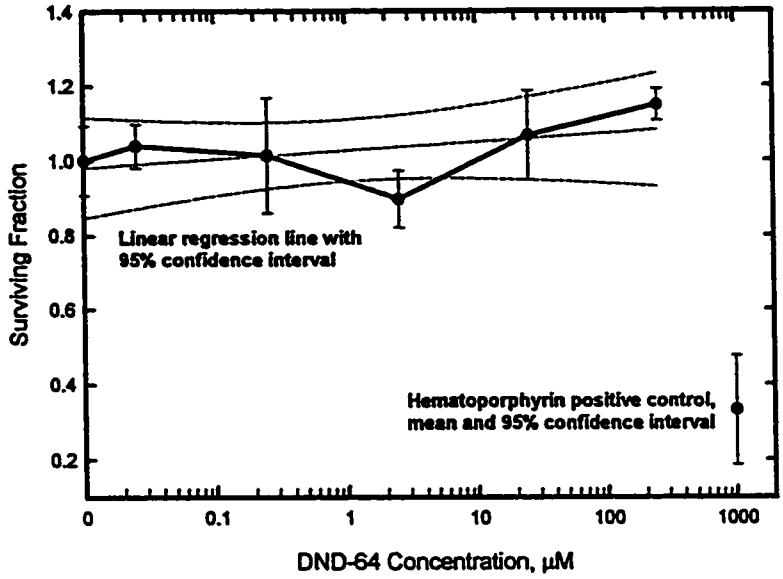
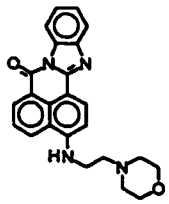


Figure 6.14. Sonodynamic activity of DND-64 in HL-60 cells given 10s insonation at 2.0W/cm^2.



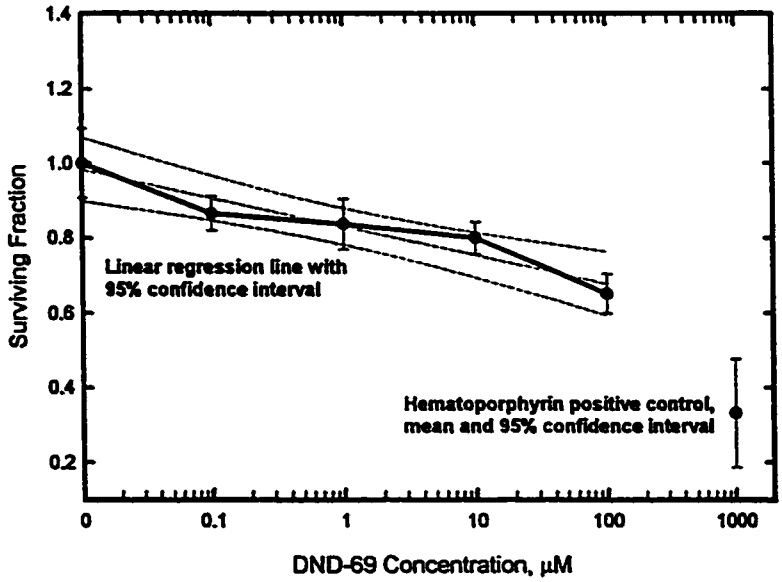
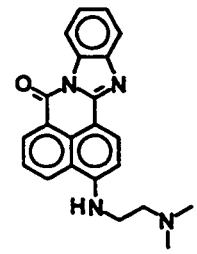


Figure 6.15. Sonodynamic activity of DND-69 in HL-60 cells given 10s insonation at 2.0W/cm^2. RPMI-based cell culture medium became saturated with DND-69 at a concentration of approximately 100μM.



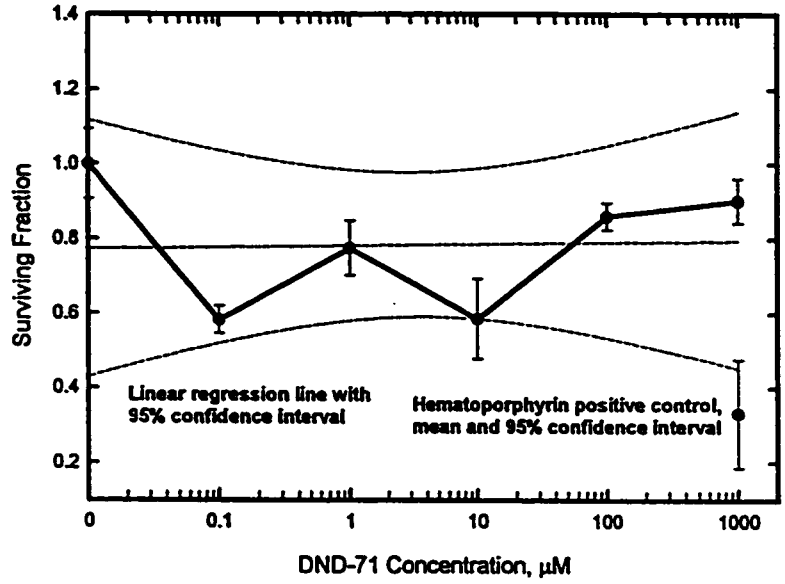
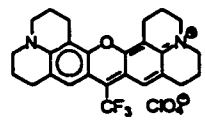


Figure 6.16. Sonodynamic activity of DND-71 in HL-60 cells given 10s insonation at 2.0W/cm^2.



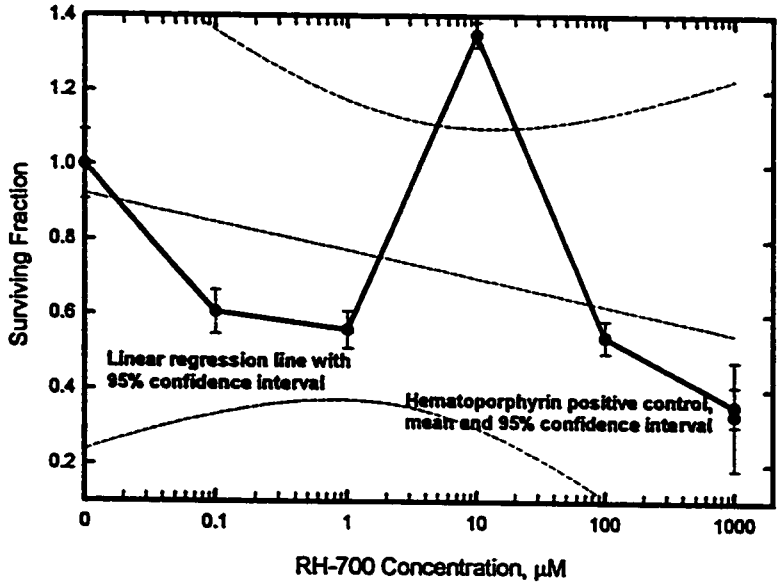
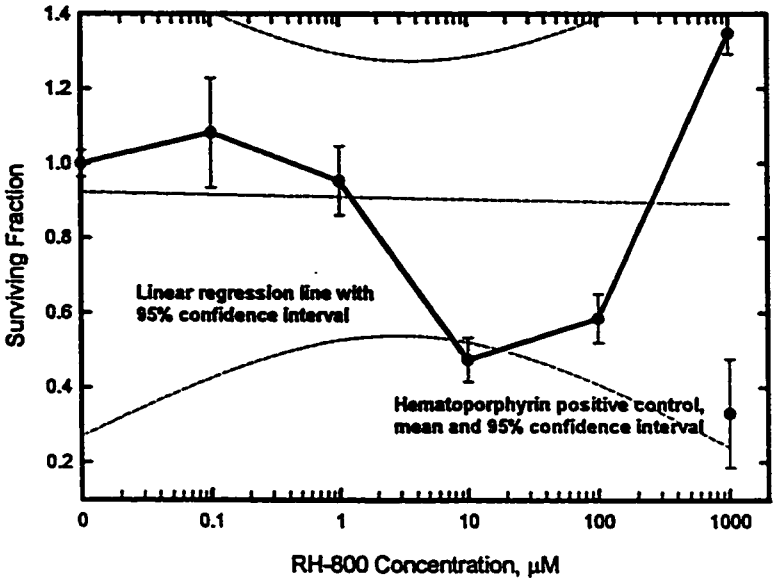


Figure 6.17. Sonodynamic activity of RH-700 in HL-60 cells given 10s insonation at 2.0W/cm^2.



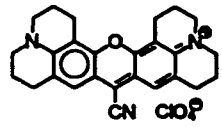
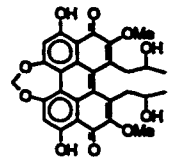


Figure 6.18. Sonodynamic activity of RH-800 in HL-60 cells given 10s insonation at 2.0W/cm^2.

III. PQP Compounds



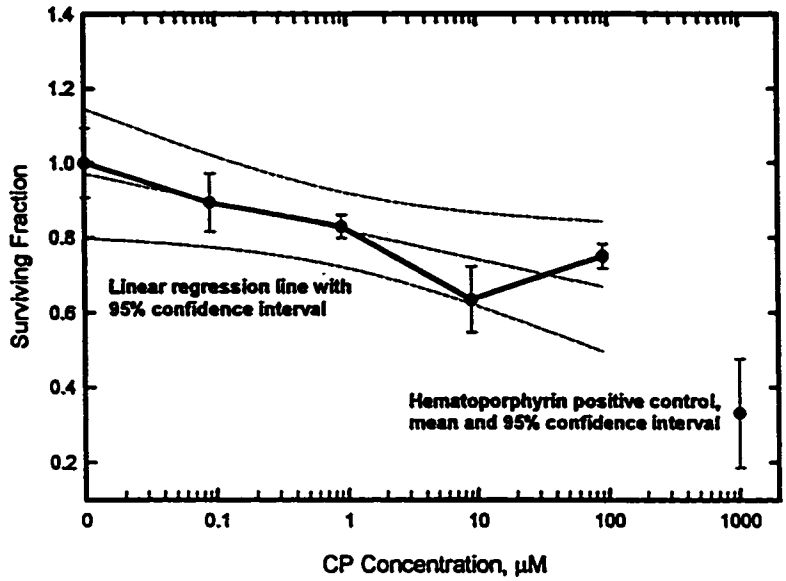


Figure 6.19. Sonodynamic activity of CP in HL-60 cells given 10s insonation at 2.0W/cm^2. RPMI based cell culture medium became saturated with CP at a concentration of approximately 90μM.

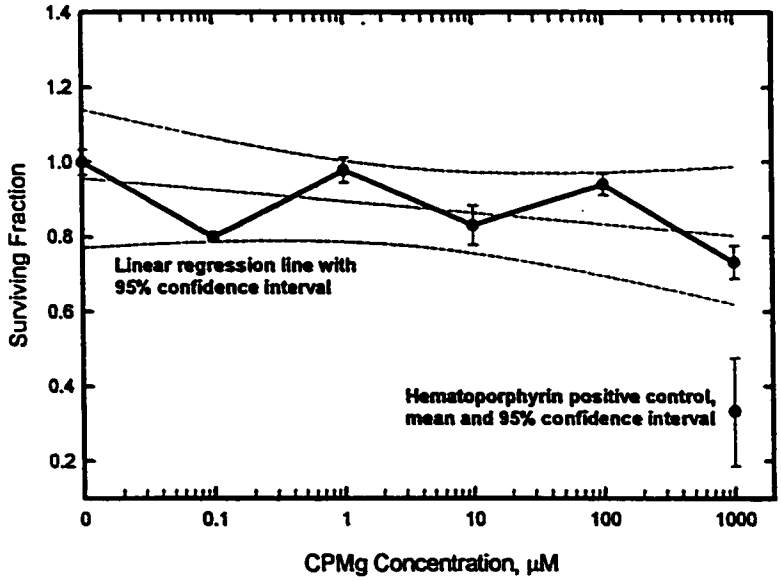
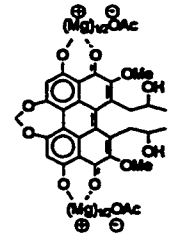


Figure 6.20. Sonodynamic activity of CPMg in HL-60 cells given 10s insonation at 2.0W/cm^2.



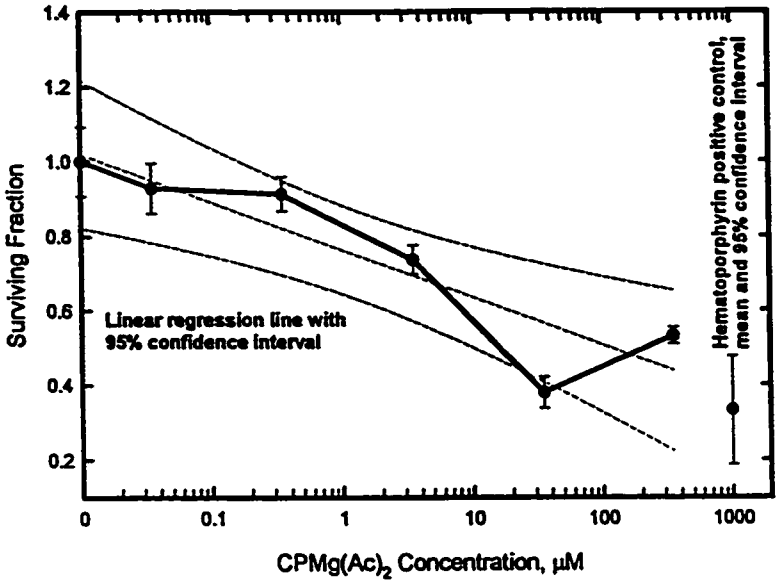
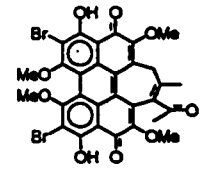


Figure 6.21. Sonodynamic activity of $CPMg(Ac)_2$ in HL-60 cells given 10s insonation at 2.0W/cm^2.



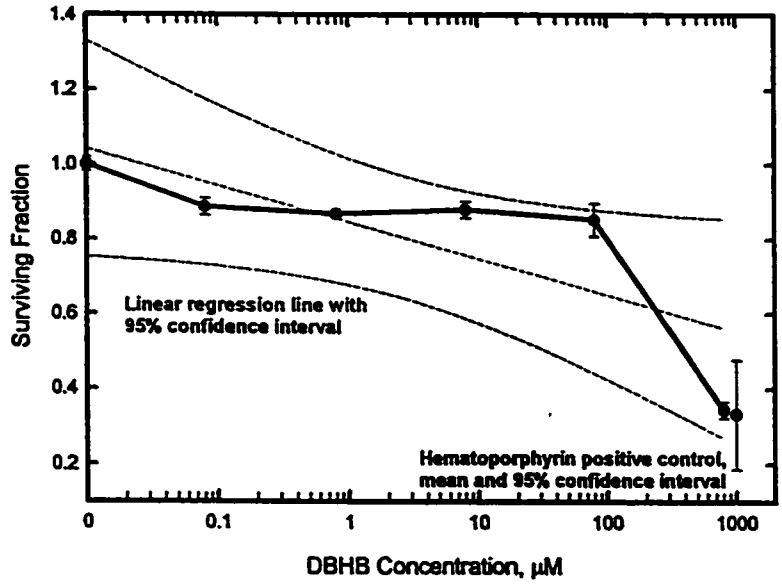
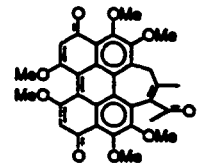


Figure 6.22. Sonodynamic activity of DBHB in HL-60 cells given 10s insonation at 2.0W/cm^2.



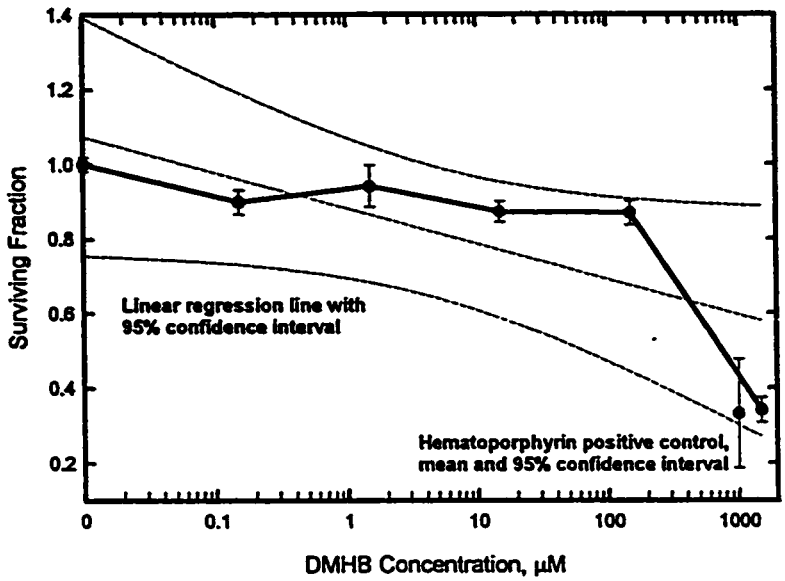
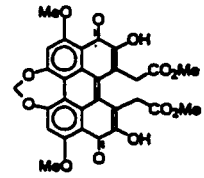


Figure 6.23. Sonodynamic activity of DMHB in HL-60 cells given 10s insonation at 2.0W/cm^2.



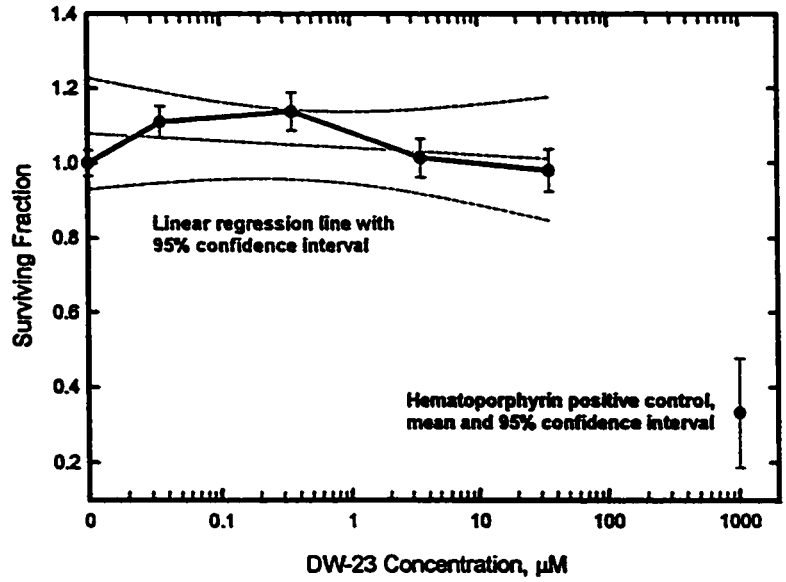
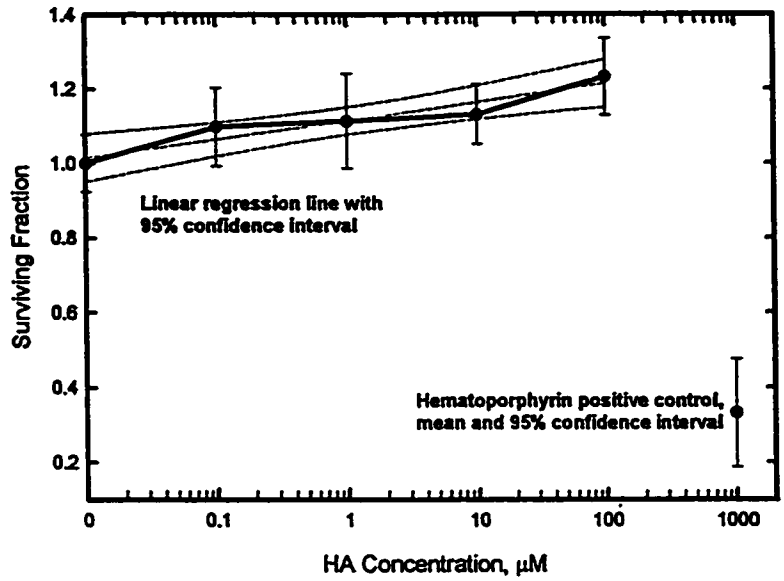


Figure 6.24. Sonodynamic activity of DW-23 in HL-60 cells given 10s insonation at 2.0W/cm^2. RPMI-based culture medium became saturated with DW-23 at a concentration of approximately 35μM.



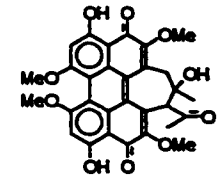


Figure 6.25. Sonodynamic activity of HA in HL-60 cells given 10s insonation at 2.0W/cm^2. RPMI-based culture medium became saturated with HA at a concentration of approximately 100μM.

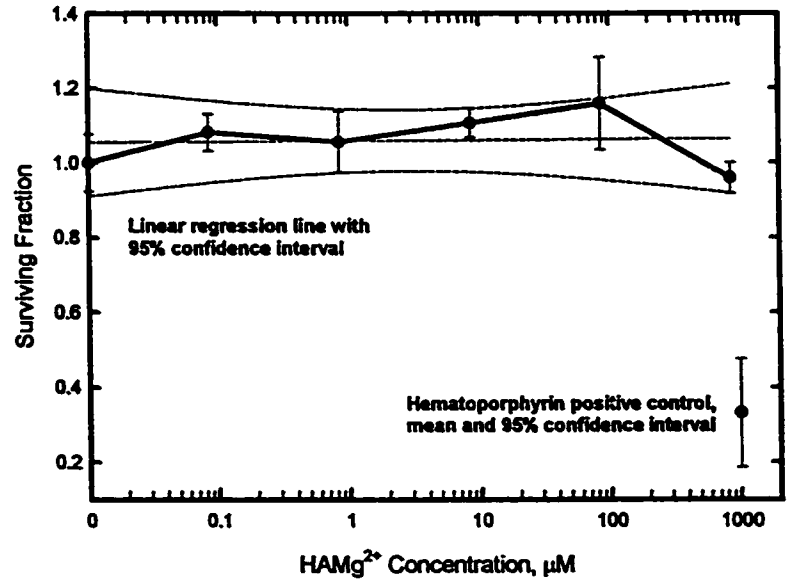
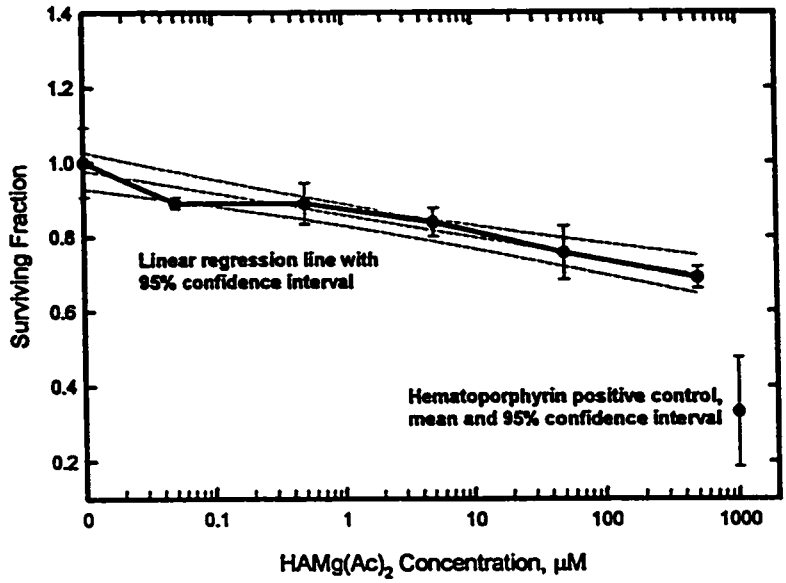


Figure 6.26. Sonodynamic activity of $HAMg^{2+}$ in HL-60 cells given 10s insonation at 2.0W/cm^2.



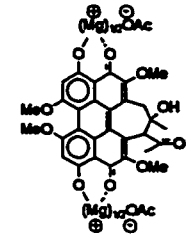


Figure 6.27. Sonodynamic activity of $HAMg(Ac)_2$ in HL-60 cells given 10s insonation at 2.0W/cm^2.

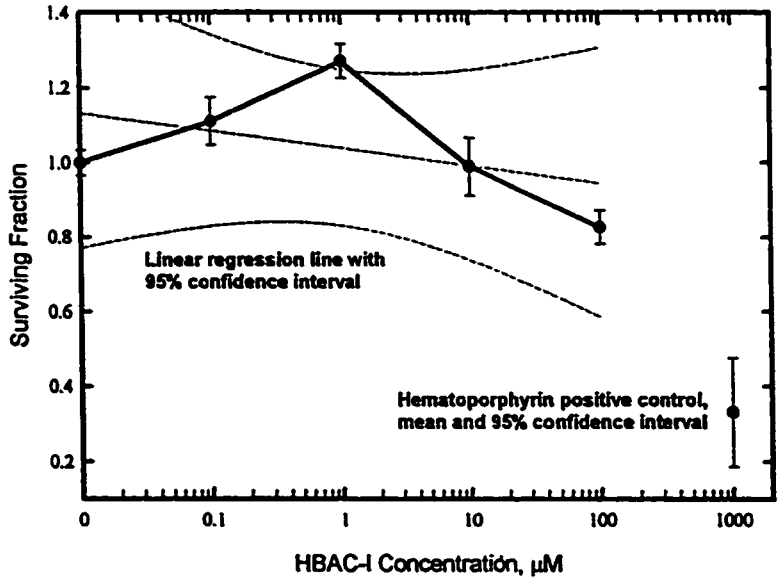


Figure 6.28. Sonodynamic activity of HBAC-I in HL-60 cells given 10s insonation at 2.0W/cm^2. RPMI-based culture medium became saturated with HBAC-I at a concentration of approximately 100μM.

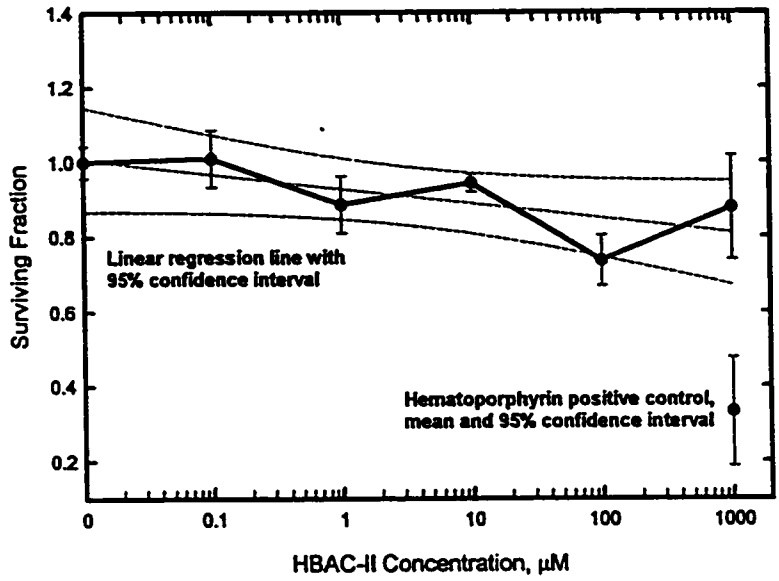
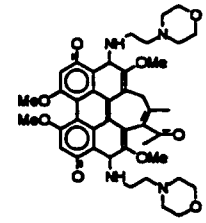


Figure 6.29. Sonodynamic activity of HBAC-II in HL-60 cells given 10s insonation at 2.0W/cm^2.



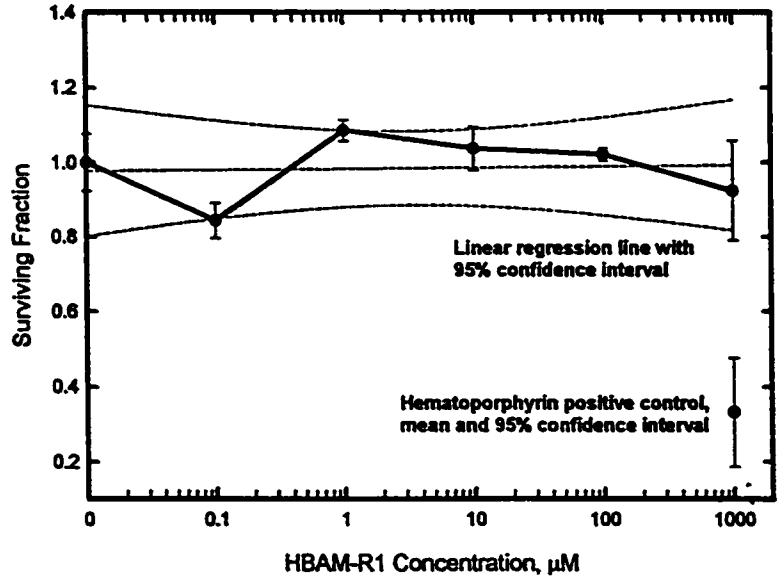
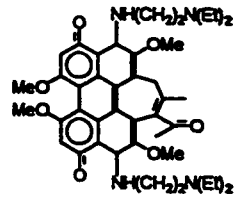


Figure 6.30. Sonodynamic activity of HBAM-R1 in HL-60 cells given 10s insonation at 2.0W/cm^2.



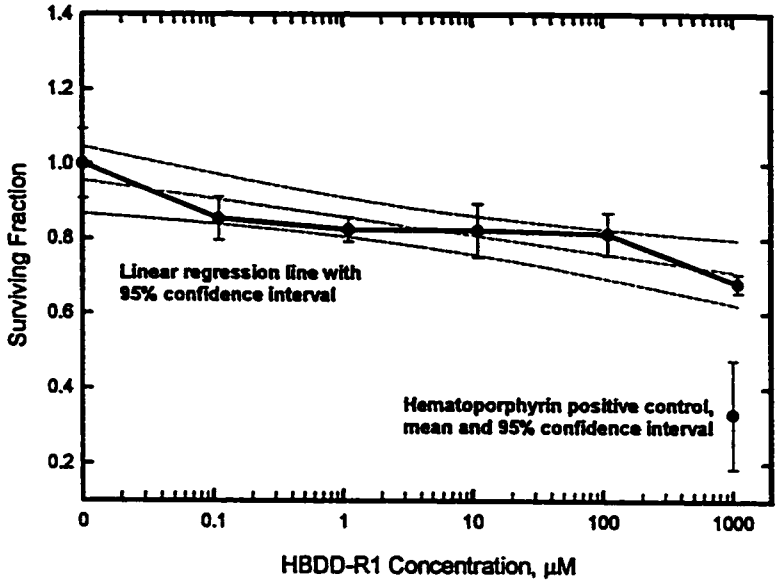
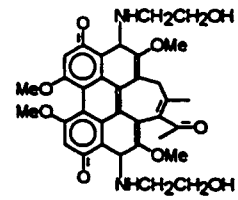


Figure 6.31. Sonodynamic activity of HBDD-R1 in HL-60 cells given 10s insonation at 2.0W/cm^2.



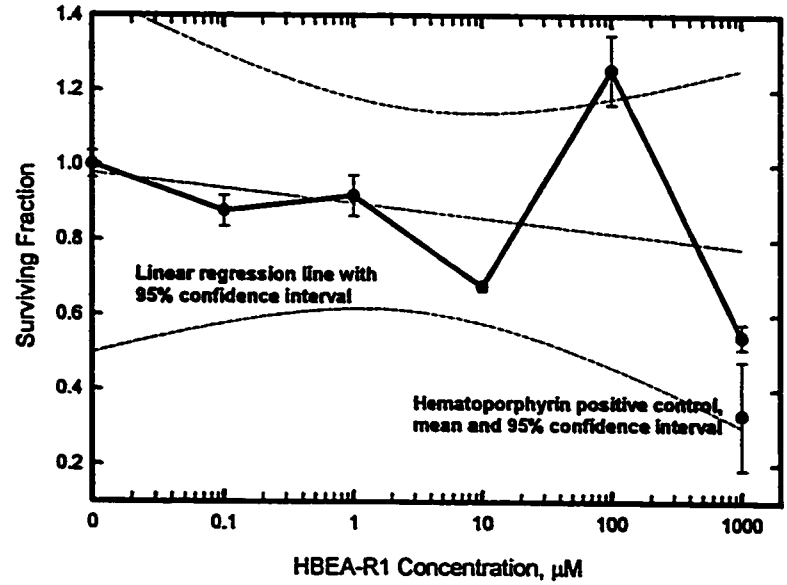


Figure 6.32. Sonodynamic activity of HBEA-R1 in HL-60 cells given 10s insonation at 2.0W/cm^2.

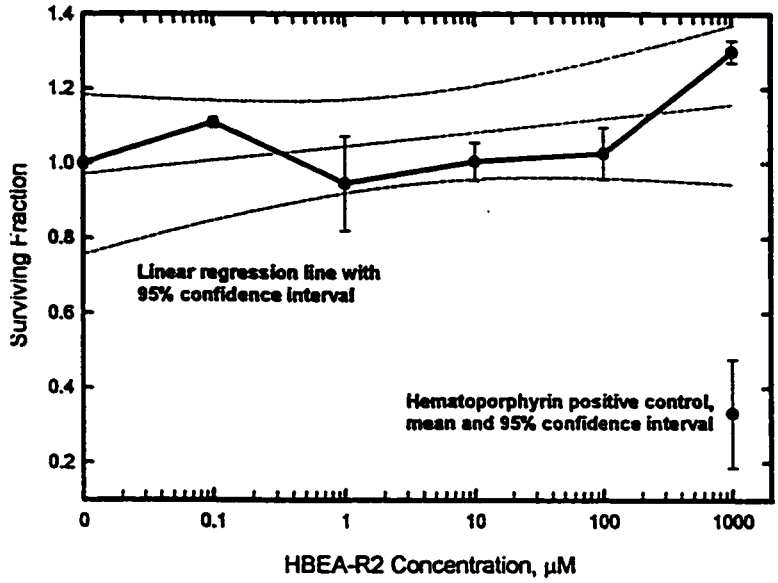


Figure 6.33. Sonodynamic activity of HBEA-R2 in HL-60 cells given 10s insonation at 2.0W/cm^2. The final point actually showed a surviving fraction of 1.79.

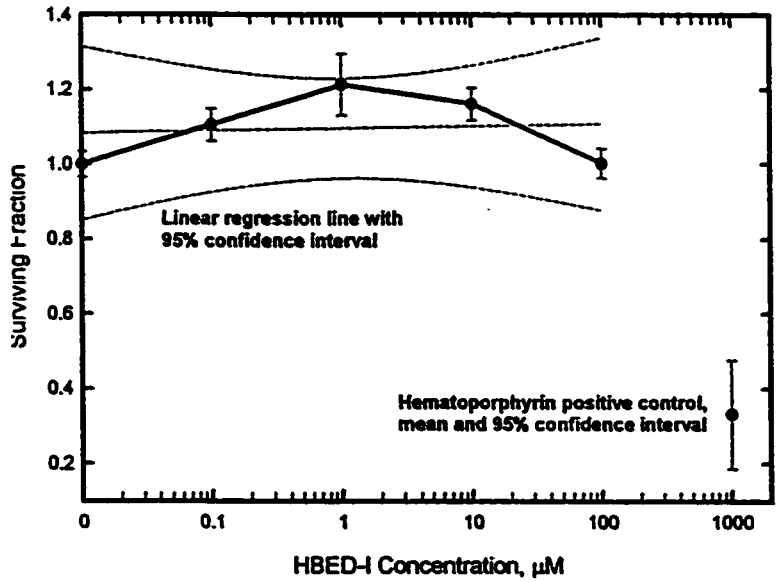


Figure 6.34. Sonodynamic activity of HBED-I in HL-60 cells given 10s insonation at 2.0W/cm^2. RPMI-based culture medium became saturated with HBED-I at a concentration of approximately 100μM.

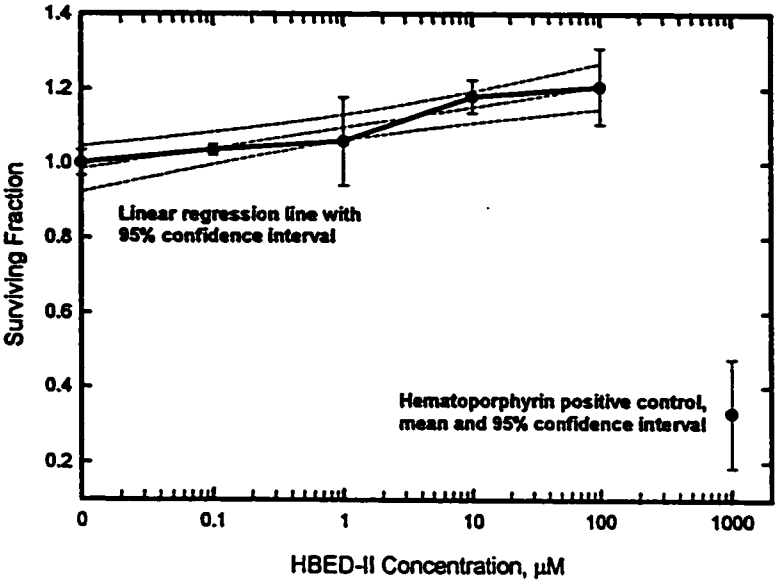


Figure 6.35. Sonodynamic activity of HBED-II in HL-60 cells given 10s insonation at 2.0W/cm^2. RPMI-based culture medium became saturated with HBED-II at a concentration of approximately 100μM.

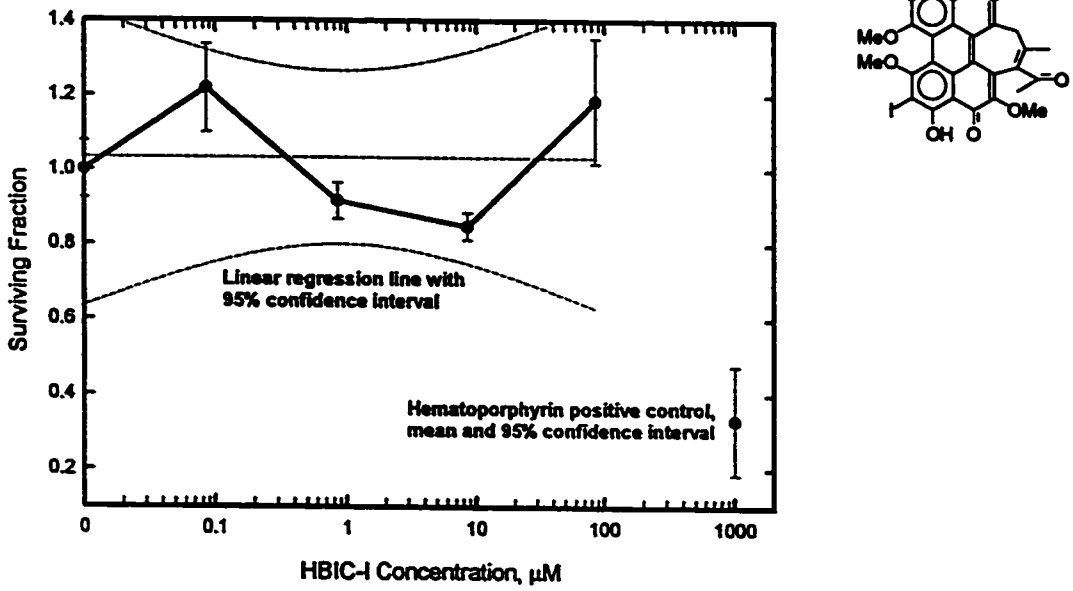


Figure 6.36. Sonodynamic activity of HBIC-I in HL-60 cells given 10s insonation at 2.0W/cm^2. RPMI-based culture medium became saturated with HBIC-I at a concentration of approximately 85μM.

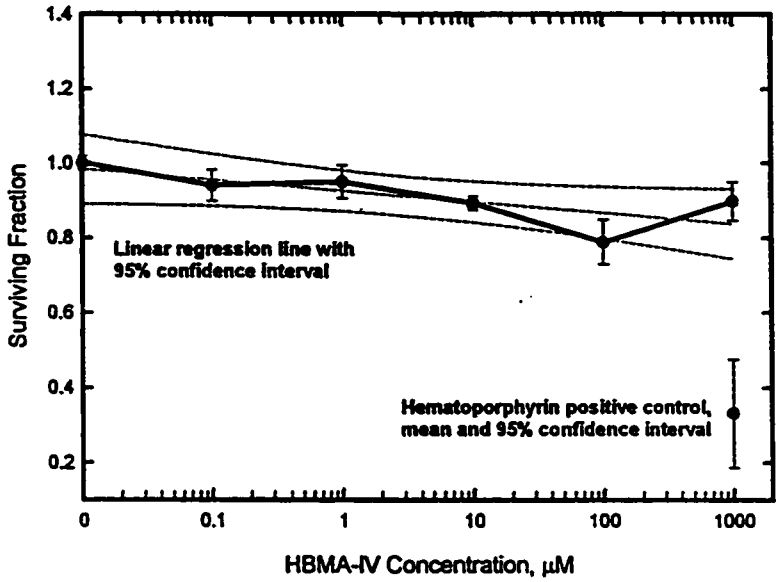
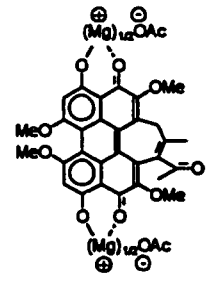


Figure 6.37. Sonodynamic activity of HBMA-IV in HL-60 cells given 10s insonation at 2.0W/cm^2.



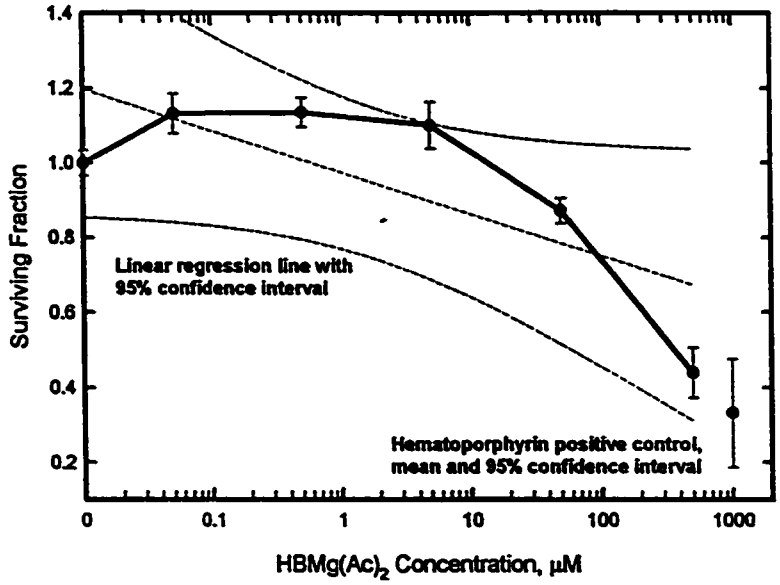
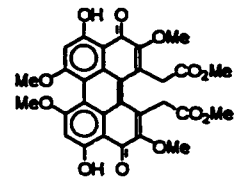


Figure 6.38. Sonodynamic activity of $HBMg(Ac)_2$ in HL-60 cells given 10s insonation at 2.0W/cm^2.



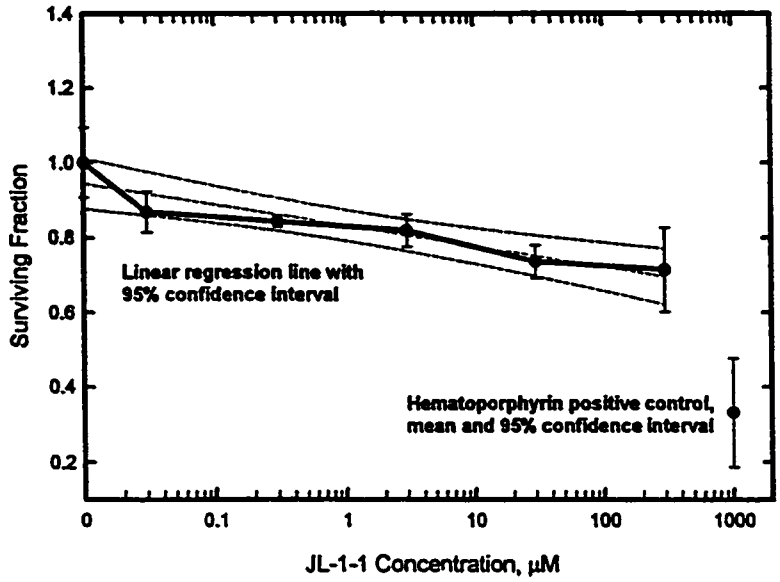


Figure 6.39. Sonodynamic activity of JL-1-1 in HL-60 cells given 10s insonation at 2.0W/cm^2.

Chapter 7

General Conclusions and Future Directions

7.1 Conclusions

Work described in this thesis represents several advances in the field of experimental sonodynamic therapy. Experimentally, the successful use of a standard physiotherapy ultrasound device instead of a uniquely constructed transducer demonstrates the feasibility of using standard equipment in the study of sonosensitization. Testing sonosensitizing compounds under standard conditions will permit better comparison of the results of different research groups. The apparatus used here was designed to transmit ultrasonic energy to solid tissue, and can be used for this purpose simply by removing the adapter cuff used for *in vitro* insonations. A further advantage of this device is that it can be used in the clinical evaluation of sonosensitizers, as it is also limited to ultrasonic intensities licensed for clinical use. Development of sonosensitizers using an apparatus meeting United States Food and Drug Administration and Health Canada guidelines for human use will permit its use throughout the development of sonosensitizing drugs. The same apparatus may be used for *in vitro*, *in vivo* and clinical testing of candidate compounds.

Thorough characterization of a particle counter-based cytotoxicity assay also represents a significant advance (Kessel *et al.*, 1995). In addition to the potential errors that may be made using dye-based and clonogenic viability assays, most such assays are labour-intensive, relatively slow and the data they generate are usually based on counts of small numbers of cells. The particle counter assay used in these studies provides a real-time estimate of

the cell density at the conclusion of insonation. It has a throughput of thirty samples per hour, and yields data based upon counts of far larger numbers of cells than is possible in other assays. The speed and reliability of this system are important contributions to experimental work in this field.

The reliability of the results obtained using the modified physiotherapy device in combination with the particle counter permitted careful study of the kinetics of cell survival in the presence of ultrasound. This study produced a mathematical model with important implications for the mechanism of immediate cell destruction induced by ultrasound. After minor modification, it is able to account for the mechanisms of sonostatic and sonodynamic compounds in the sensitization of cells to immediate ultrasonic destruction.

Potential difficulties that may arise from the use of common solvents for the administration of sonosensitizers have been addressed by examining four of these solvents for the magnitude of their sonotoxic effects and for their possible mechanism. Tests of the concentration effects of these solvent sonosensitizers showed that their effects were not unacceptably great, and that PEG-300 and DMSO showed much smaller sonotoxic effects than DMF and MMF. Further study of these solvents in light of the mathematical model proposed for ultrasound-induced cell death showed that their mechanism of action may be different from that of sonosensitizers with clinical potential.

Finally, this work has identified six compounds originally examined for their photosensitizing potential which also have sonosensitizing activity. These compounds have clinical properties that are more favourable than those of porphyrin-derived sonosensitizers (Estey *et al.*, 1996), and exhibit sonosensitizing activity of the same magnitude as Hp. One of these compounds exhibits its maximum sonotoxic effect at a concentration approximately one third as great as that of Hp. Two have a range of two decades of concentration over which their sonotoxic effect occurs, which may enable easier adjustment of their clinical effects.

7.2 Future Directions and Possible Limitations

This work points to certain further lines of investigation beyond the scope of this thesis. Two further experiments would complement those presented in Chapter 5, which explain the action of solvents in increasing immediate sonotoxicity. The first would concern cells treated with a photosensitizer with weak sonosensitizing properties. Simultaneous insonation and irradiation with visible light could be used to construct survival curves in the manner presented here for purely sonodynamic systems. Such a system guarantees constantly increasing damage induced by the photosensitizer, and the survival kinetics of the system will show intrinsically nonlinear behaviour if the mathematical model presented in this thesis is correct. The second experiment would test a strong sonosensitizer, such as the gallium-porphyrin complex ATX-70, under purely sonodynamic conditions. Should such experiments yield survival

functions of similar form to that of the combination of sono- and photo-dynamic treatments, it would be consistent with sonoluminescent activation of sonosensitizers. This would provide further evidence of a link between photodynamic and sonodynamic activities, and perhaps provide a further criterion for the development of clinically useful sonosensitizing antitumour drugs.

It must be noted, though, that meaningful application of the survival model may be prevented by two factors. First, while the assay system presented in this thesis is rapid and reliable, the noise inherent in the system is approximately ten *per cent.* of initial survival, as measured by the standard deviation of residual values from fitted curves. Second, HL-60 cells are composed of two subpopulations with distinctly different survival half-lives in the presence of ultrasound.

The signal-to-noise ratio[##] (S/N) through the sonoresistant portion of the curve, which ranges from 5dB to 15dB in these studies, permits accurate modeling of its survival pattern. This does not hold for the sonosensitive subpopulation, whose S/N nears 10dB at the start of insonation and rapidly decreases to negative values. S/N ratios up to 2.5dB at best permit marginal signal detection (Lewis, 1997), and because of this the survival data cannot be modeled

[##] The signal-to-noise ratio is defined as S/N = $20 log_{10}$(Signal strength/Noise strength), and is measured in decibels (dB).

accurately. At such low S/N, the noise overwhelms the signal and the uncertainty of the parameters estimated for the model is very large.

This restricts the utility of this model for the detection nonlinearity in survival kinetics, since detection of nonlinearity is dependent upon significant difference of the ε terms from zero (see Section 4.3). The high S/N through the sonoresistant decay makes tests of nonlinearity reasonably sensitive for this subpopulation, since the small relative errors for the test parameter ε_2 increase the significance of observed values of ε_2. The low S/N through the sonosensitive decay results in a large relative error estimate for the test parameter, ε_1. Such a circumstance decreases the statistical significance of any observed value of ε_1, and greatly reduces the ability of this model to distinguish intrinsically nonlinear behaviour from intrinsically linear behaviour. Until survival assays are developed that have substantially less noise (less than five *per cent.* of initial survival), these deficiencies limit use of this model to evaluation of sonotoxic mechanisms in sonoresistant subpopulations.

Last, the six novel sonosensitizers identified in this work must be more carefully characterized. The screen used here was designed to identify compounds possessing sonosensitizing activity using only small quantities of the compounds. Since the quantities of compounds available were vanishingly small, many were consumed completely in a single test. Those that did show sonodynamic activity can now be synthesized in greater quantities to enable

confirmation of the observed activity, evaluation of ultrasound-induced singlet oxygen production by EPR spectroscopy, and eventual evaluation of clinical and pharmacokinetic properties.

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Appendix

S-Plus Code and Output for Curve Fitting and Lack-of-Fit Analysis

```
> ### DMSO2 DATA
t.sc <- DMSO2[[1]]
> S.sc <- DMSO2[[2]]   # Get starting values
> a.start <- 0.68
> alpha.start <- 0.09
> beta.start <- 0.59
> starting.values <- list(a.start, alpha.start, beta.start)
> names(starting.values) <- c("a", "alpha", "beta")
> parameters(DMSO2) <- list(a = a.start, alpha = alpha.start, beta =
   beta.start)
> fit.sc <- nls(S ~ a * exp( - alpha * t) + (1 - a) * exp( - beta * t), DMSO2,
   start = starting.values, trace = T)
0.657719 : 0.68 0.09 0.59
0.0407184 : 0.504917 0.11166 0.858987
0.0316312 : 0.517082 0.127966 0.796053
0.0315822 : 0.520573 0.129938 0.79807
0.0315822 : 0.520956 0.130033 0.798683
> print(summary(fit.sc))

Formula: S ~ a * exp( - alpha * t) + (1 - a) * exp( - beta * t)

Parameters:
        Value Std. Error t value
    a 0.520956  0.0642590 8.10714
alpha 0.130033  0.0150844 8.62037
 beta 0.798683  0.1147280 6.96153

Residual standard error: 0.029619 on 36 degrees of freedom

Correlation of Parameter Estimates:
          a alpha
alpha 0.935
 beta 0.936 0.825
> par(mfcol = c(3, 1))
> plot(x = t.sc, y = S.sc, xlab = "Insonation Time", ylab = "Surviving Fraction",
   type = "p")
> title(sub = "DMSO 2: Data and Fits")
> lines(x = t.sc, y = fit.sc$fitted.values)
> scatter.smooth(x = fit.sc$fitted.values, y = fit.sc$residuals, xlab =
   "Fitted Values", ylab = "Residuals")
> title(sub = "DMSO 2: Residuals vs. Fitted Values")
> scatter.smooth(x = t.sc, y = fit.sc$residuals, xlab = "Fitted Values", ylab =
   "Residuals")
> title(sub = "DMSO 2: Residuals vs. Insonation Time (s)") ## LACK OF FIT
> time <- t.sc
> frac <- S.sc
> n <- length(frac)
> p <- length(fit.sc$parameters)
> unique.times <- unique(time)
> SSPE <- 0
> df.PE <- 0
> for(t in unique.times) {
   S <- frac[time == t]
   SSPE <- SSPE + sum((S - mean(S))^2)
   df.PE <- df.PE + length(S) - 1
}
> SSE <- sum(fit.sc$residuals^2)
> SSLOF <- SSE - SSPE
> df.SSE <- n - p
> df.LOF <- df.SSE - df.PE
> df <- c(df.LOF, df.PE, df.SSE)
> MSPE <- SSPE/df.PE
> MSLOF <- SSLOF/df.LOF
> MSE <- SSE/df.SSE
> F.lof <- MSLOF/MSPE
> p.lof <- 1 - pf(F.lof, df.LOF, df.PE)
> anova <- cbind(c(SSLOF, SSPE, SSE), df, c(MSLOF, MSPE, MSE), c(F.lof, -99, -99
   ), c(p.lof, -99, -99))
> dimnames(anova) <- list(c("Lack of Fit", "Pure Error", "Error"), c("SS", "df",
   "MS=SS/df", "F", "p"))
> anova <- round(anova, 4)
> anova[anova == -99] <- c("  ")


> cat("\n", "                  LACK OF FIT", "\n")

                  LACK OF FIT
> print.matrix(anova, quote = F)
               SS df MS=SS/df      F     p
Lack of Fit 0.0098 8  0.0012 1.5727 0.178
 Pure Error 0.0218 28 0.0008
      Error 0.0316 36 0.0009
```