



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

**Photodynamic therapy against
oesophageal cancer. *In Vitro* testing
of potential new agents.**



Submitted by

Gisela Vaz

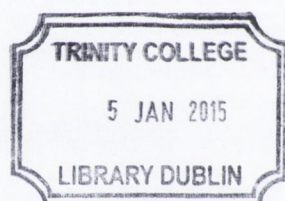
M.Sc. (Molecular Medicine), University of Cranfield, United
Kingdom

A thesis submitted to the University of Dublin, Trinity College for
degree of

Doctor of Philosophy

University of Dublin, Trinity College

2014



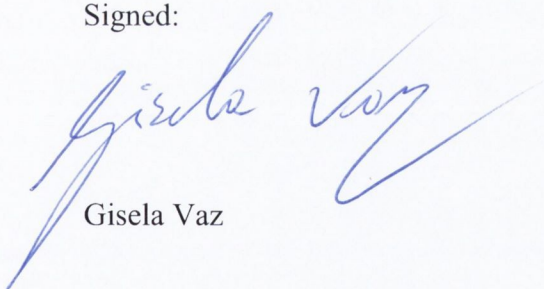
Thesis 10742

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Signed:

A handwritten signature in blue ink, appearing to read 'Gisela Vaz', with a long, sweeping flourish extending to the right.

Gisela Vaz

Summary

Oesophageal malignancies are one of the leading causes of death in the Western World. The incidence of these diseases is currently on the rise alternative but powerful therapies are being developed against them, an example of these is photodynamic therapy (PDT) which is an emerging treatment against oesophageal cancer. However, it is still a long way from being widely used as an alternative to the more conventional therapies. The most commonly used photosensitisers are based on porphyrins and their derivatives yet those in clinical use still show many side effects and issues such as prolonged patient phototoxicity.

In order to test a broad range of potential new photosensitisers a novel single step assay approach to screen a library of PDT compounds was developed in two relevant oesophageal cancer cell lines (OE21 and SKGT-4); the HCS data was evaluated and validated by performing comparative studies using a conventional colorimetric assay (MTT). Utilizing high content screening and analysis (HCS) technologies several robust cellular parameters were identified, which were used to determine the phototoxic effects of porphyrin compounds that have been developed as potential anticancer agents directed against oesophageal carcinoma. To demonstrate the proof-of-principle of this approach a study using five porphyrin based compounds was performed utilizing OE21 cells. The measurable outputs from these early studies were then evaluated by performing a pilot screen using a set of 22 compounds. The results were evaluated and validated by performing comparative studies using a colorimetric assay (MTS) in three oesophageal cell lines (OE21, SKGT-4 and OE33). The studies demonstrated that the HCS assay offers significant advantages over and above

the currently used methods (directly related to the intracellular presence of the compounds by analysis of their integrated intensity and area within the cells). A high correlation was found between the HCS and MTT data. However, the HCS approach provides additional information that allows a better understanding of the behaviour of these compounds when interacting at the cellular level. This is the first step towards an automated high-throughput screening of photosensitiser drug candidates and the beginnings of an integrated and comprehensive quantitative structure action relationship (QSAR) study for photosensitiser libraries.

Using HCS technologies and MTT a library of glycosylated porphyrins (glycoporphyrins) was evaluated for their photodynamic therapy activity against the oesophageal squamous-cell carcinoma cell line OE21 *in vitro*. It was found that all compounds tested were taken up by the cells but showed no phototoxicity under the conditions tested; this was most likely due to the fact that these molecules did not generate sufficient amounts of singlet oxygen to damage the cells at a significant extent.

A third library of porphyrins linked to the anti-inflammatory ibuprofen was also tested, these compounds showed no phototoxicity or accumulation in the cells. Yet, these molecules highlighted one of the major limitations of the HCA assay where the fluorescent compounds were measured by the automated imaging system as being inside by the cells, however, they were simply precipitated onto the outside of the cells tested.

A fourth library of phosphoporphyrins was tested using the newly developed HCA method; this showed that only two of the five compounds showed any

cytotoxicity yet offered no extra therapeutic effect when compared to the clinically approved Temoporfin.

In connection with our interest in PDT of oesophageal malignancies and with recent advance on the generation of three dimensional structures in a laboratory setting, a new spheroid generating medium Happy Cell[®] was used to cultivate oesophageal 3D spheroids *in vitro* and these were used to screen porphyrins as PDT agents.

We were able to consistently generate spheroids using OE21 and OE33 cells. In addition an inactivation protocol, using the provided inactivation solution, was optimized while sustaining optimal cell viability. Four different photosensitisers were also tested revealing that the spheroids, were more resistant to the action of PDT. We were also able to establish a protocol to fix these spheroids for long term preservation and further staining for imaging using high content and/or confocal systems.

Leading from our established HCS assay, two small libraries of small molecules were assessed as potential fluorophores for use in HCS analysis, phenanthroline derivatives and their europium^{III} and zinc^{II} complexes. Phenanthroline-based small molecules appear to localize in the endoplasmic reticulum and their potential as fluorescent probes was evaluated. The materials were quickly taken up by living cells and showed remarkable cytotoxicity towards cancer cells. Novel europium^{III} and zinc^{II} complexes were shown to be stable in cell culture media and first images were taken after a 20 min incubation time at a very low concentration range (0.7–1.6 μM).

Parts of this thesis were published in

Vaz GMF, Paszko E, Davies AM, Senge MO: High Content Screening as High Quality Assay for Biological Evaluation of Photosensitizers in Vitro. PLOS One, 2013. 8(7): e70653.

Paszko E, Vaz GM, Ehrhardt C, Senge MO: Transferrin conjugation does not increase the efficiency of liposomal Foscan during in vitro photodynamic therapy of oesophageal cancer. Eur J Pharm Sci 2013; 48:202-210.

Daly R, Vaz G, Davies AM, Senge MO, Scanlan EM: Synthesis and biological evaluation of a library of glycoporphyrin compounds. Chem Eur J 2012; 18:14671-14679.

Sergeeva NN, Donnier-Marechal M, Vaz G, Davies AM, Senge MO: Synthesis and evaluation of the europium(III) and zinc(II) complexes as luminescent bioprobes in high content cell-imaging analysis. J Inorg Biochem 2011; 105:1589-1595.

Sergeeva NN, Donnier-Marechal M, Vaz GM, Davies AM, Senge MO: Simple but powerful: phenanthroline-based small molecules for cellular imaging and cancer screening. Bioorg Med Chem Lett 2011; 21:4385-4388.

Parts of this this thesis were presented in

Vaz, G (2013): High Content Analysis: A gateway to the development of photodynamic drugs. IMM, TCD Monday talk.

Vaz, G.; Pazko, E.; Davies, A.; Senge, M. O. (2011): A high content approach to the development of novel photosensitisers. FEBS Advanced Lecture Course on Translational Cancer Research. September 27- October 4, Algarve, Portugal.

Vaz, G.; Pazko, E.; Davies, A.; Senge, M. O. (2011): Research and Development of New Photosensitisers: A High Content Approach. EACR Anticancer Agents Research Congress. October 13-16, 2011, Antalya, Turkey. Abstract book.

Vaz, G.; Pazko, E.; Davies, A.; Senge, M. O. (2011): A high content approach to the development of novel photosensitisers. In: 14th Congress of the European Society for Photobiology. September 1 - 6, 2011, Geneva, Switzerland. Abstract book.

Vaz, G.; Paszko, E.; Davies, A.; Ehrhardt, C.; Senge, M. O. (2010): A high content analysis method for the research and development of novel Photosensitisers. 13th Annual Meeting of The Institute of Molecular Medicine, 32/33, Dublin, Ireland, Abstract Book.

Sergeeva, N.; Donnier-Marechel, M.; Vaz, G.; Davies; M.; Senge, M. (2010):

Small molecules for fluorescence imaging and cancer diagnostics. 13th Annual Meeting of The Institute of Molecular Medicine, 32/33, Dublin, Ireland, Abstract Book.

Vaz, G.; Paszko, E.; Davies, A.; Ehrhardt, C.; Senge, M. O.(2009): Liposomal formulations of photosensitisers and a high content screening approach for the advancement of photodynamic therapy (PDT). Recent Advances in Synthesis and Chemical Biology VIII, CSCB Symposium 2009, 18/12, Dublin, Ireland, Abstract Book.

Vaz, G.; Paszko, E.; Davies; Senge, M. O. (2009): A high content screening approach for the advancement of photodynamic therapy (PDT). 12th Annual Meeting of The Institute of Molecular Medicine, 28, Dublin, Ireland, Abstract book.

Vaz, G.; Paszko, E.; Davies, A.; Ehrhardt, C.; Senge, M. O. (2009): Liposomal formulations of photosensitizers for photodynamic cancer therapy. 12th Annual Meeting of The Institute of Molecular Medicine, 29, Dublin, Ireland, Abstract book.

Acknowledgements

I would like to thank everyone in the Senge group (past and present members) for providing me with advice, support and most of all compounds to test, especially Ms. Rosine Petitdemange for her interest in PDT and for all her help. Also a big thank you to everyone in the IMM, especially Dr. Edyta Paszko and Dr. Natalyia Sergeeva for their help, advise and happiness in general.

A special thank you to my supervisor Professor Senge for many years of new experiences and my co-supervisor Dr. Anthony Davies whose presence and guidance was priceless.

A big thank you to Ms Sarah-Lu Ryan for working with me and helping me during uncertain times and possibly doomed experiments.

Thank to you to my family and friends for their support.

To my husband Micael Gomes my rock during this PhD, I could have not done it without you, thank you for putting up with me!

I would finally like to acknowledge and thank the funding bodies SFI and FCT (through the POPH program) for financially supporting my project.

Table of Contents

SUMMARY	5
ACKNOWLEDGEMENTS	12
TABLE OF CONTENTS	13
ABBREVIATIONS	15
CHAPTER ONE: INTRODUCTION.....	19
1.1 PHOTODYNAMIC THERAPY.....	19
1.2 PDT AND OESOPHAGEAL CANCER	27
1.3 PHOTOSENSITISERS	30
1.4 LIGHT SOURCES.....	34
1.5 HIGH CONTENT TECHNOLOGY	37
1.6 3D TUMOUR MODELS AND THEIR RELEVANCE IN THE FIELD OF DEVELOPMENT OF NEW THERAPEUTICS	47
1.7 LUMINESCENCE BIOPROBES.....	51
1.8 AIMS AND OBJECTIVES	56
CHAPTER TWO: MATERIALS AND METHODS.....	57
2.1 CELL LINES.....	57
2.2 CELL CULTURE	57
2.3 HIGH CONTENT SCREENING ASSAY DEVELOPMENT AND SCREEN OF PORPHYRIN LIBRARIES.....	58
2.3.1 Treatments	58
2.3.2 Illumination protocol.....	59
2.3.3 Cell proliferation assay	59
2.3.4 High content imaging and analysis	60
2.4 ASSAY APPLICATION TO VARIOUS PORPHYRIN LIBRARIES	62
2.5.1 Assessment of spheroid formation.....	62
2.5.2 Viability of spheroids following PDT	63
2.5.3 Fixation and further staining	63

2.6 ASSESSMENT OF SMALL MOLECULES AS POTENTIAL FLUOROPHORES	64
2.6.1 <i>Live cell testing</i>	64
2.6.2 <i>Fixed cell testing</i>	64
2.6.3 <i>Confocal imaging</i>	65
2.7 CONFOCAL IMAGE COLLECTION	65
2.8 STATISTICAL ANALYSIS	65
 CHAPTER THREE: RESULTS AND DISCUSSION	 67
3.1 IN CELL 1000 EXPERIMENTS	67
3.2 HIGH CONTENT SCREENING ASSAY DEVELOPMENT.....	68
3.2.1 <i>Cell proliferation</i>	69
3.2.2 <i>High content analysis</i>	70
3.2 APPLICATION OF HCS PROTOCOL TO LIBRARIES OF PORPHYRINS	84
3.3.1 <i>Proof of concept: Small library of five porphyrins</i>	84
3.3.2 <i>Library of twenty two porphyrins</i>	93
3.3.3 <i>Library of novel porphyrins linked to anti-inflammatory molecules</i>	103
3.3.4 <i>Library of novel glycoporphyrins</i>	111
3.3.5 <i>Library of novel phosphorous porphyrins</i>	121
3.4 DEVELOPMENT OF OESOPHAGEAL 3D SPHEROIDS AND ASSESSMENT OF THEIR SUITABILITY FOR SCREENING PORPHYRINS.....	133
3.5 ASSESSMENT OF A LIBRARY OF COMPOUNDS AS POSSIBLE CELLULAR FLUOROPHORES	144
3.5.1 <i>Phenanthroline-based small molecules</i>	146
3.5.2 <i>Phenanthroline EuropiumIII and ZincII complexes</i>	153
 CHAPTER FIVE: CONCLUSIONS AND FUTURE PERSPECTIVES....	 157
REFERENCES	163

Abbreviations

$^1\text{O}_2$ – singlet oxygen

$^3\text{O}_2$ – triplet oxygen

3P^* – photosensitiser in triplet excited state

AlPcSz – disulfonated aluminum phthalocyanine

ALA – aminolevulinic acid

ANOVA - analysis of variance

BPD-MA – benzoporphyrin derivative monoacid

BrdU – 5-bromo-2'-deoxyuridine

CFP – cyan fluorescent protein bottom

COX – cyclooxygenase

DAPI – 4,6-diamidino-2-phenylindole

DMSO – dimethyl sulfoxide

ECACC – European Collection of Cell Cultures

EDTA – ethylenediaminetetraacetic acid

EMA – Europe the European Medicines Agency

ER – endoplasmic reticulum

FBS – fetal bovine serum

EtOH – ethanol

FDA – Food and Drug Administration

GERD – gastroesophageal reflux disease

GI – gastro intestinal

HBSS – hanks buffered salt solution

HCA – high content analysis

HCS – high content screening

Hp – hematoporphyrin

HpD – hematoporphyrin derivative

LED – light emitting diode

Ln – lanthanide

MeOH – methanol

*m*THPC – *m*-tetrahydroxyphenylchlorin

MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NSAID – non-steroidal anti-inflammatory drug

OPD – optical penetration depth

P⁻ – reduced photosensitiser

P⁺ – oxidized photosensitiser

PBS – phosphate buffer saline

PDT – photodynamic therapy

Pen/Strep – penicillin/streptomycin

P+Et – 40% Propylene glycol plus 40% Ethanol

PFA – paraformaldehyde solution

PMS – phenazine methosulfate

PS – photosensitisers

PP – phosphorous porphyrin

S – substrate

S^+ – oxidized substrate

SnEt_2 – tin ethyl etiopurpurin

$S(O)$ – oxygen adduct of a substrate

Temo – temoporfin

THF – tetrahydrofuran

YFP – yellow fluorescent protein

Zn – zinc

CHAPTER ONE:

Introduction

1.1 Photodynamic therapy

Photodynamic therapy (PDT) is a form of treatment against cancer and other non-malignant diseases [1, 2]. It involves the administration of a photoactivable compound that, when excited by light at a specific wavelength and in the presence of oxygen, results in the production of highly reactive oxygen species, such as, singlet oxygen ($^1\text{O}_2$) [1]. This $^1\text{O}_2$ oxidizes several biomolecules and is therefore responsible for the cell death that ensues from these types of treatments. The compounds most commonly used as photosensitisers (PS) are those based on porphyrins (and their derivatives) [1]. PDT is a perfect example of the successes of basic research translated into clinical practise [1].

Light has been used for thousands of years to treat disease, the ancient Egyptians, Chinese and Indians used psolarens and light to treat illnesses like leucoderma (vitiligo) and psoriasis [1, 3, 4]. But it was not until the beginning of the 20th century that the Western scientific community learned about the connection between light, photoactivable compounds and the treatment of disease [4]. Oscar Raab, a medical student working under Hermann von Tappeiner [4-6], reported the lethal effect of acridine red and light in the paramecium *Infusoria* [3]. In this accidental discovery Raab was able to prove that the lethal effect of acridine red plus light was greater than when acridine red or light were applied separately to the paramecia [4].

In 1903, von Tappeiner and Jesioneck reported the first results of PDT for the treatment of skin cancer, using eosin as a PS [1, 6]. Other important landmarks in the story of PDT in western medicine include:

- Hausman reported the photobiological effects of porphyrins in 1908 [1]
- Dr. Meyer-Betz reported the first injection a porphyrins into humans (himself) [1] with extraordinary consequences in 1912 [1].

In the mid 1970's Dr. Tom Doherty initiated what would be the “rebirth” of the scientific interest in PDT [1]. In the same year Kelly *et al.* reported the cure of bladder cancer in mice by PDT [7]. Today, PDT is a well-established, simple, cost effective and efficient therapy used in the clinic for the treatment of some cancers (solid tumours), pre-cancerous lesions, and other non-malignant afflictions such as age related macular degeneration [1] and acne [8].

PDT, in the context of cancer treatment, is based on the application (injection in the case of solid internal tumours, topical application in the case of skin malignancies or external tumours) of a PS; time is then allowed to pass in order for the PS to accumulate in the neoplastic tissue and the site is then irradiated with light (preferably in the red region, due to its deeper penetration into tissues[9]) and cell death ensues (Fig. 1.1.1 and 1.1.2), sparing adjacent healthy tissues [10].

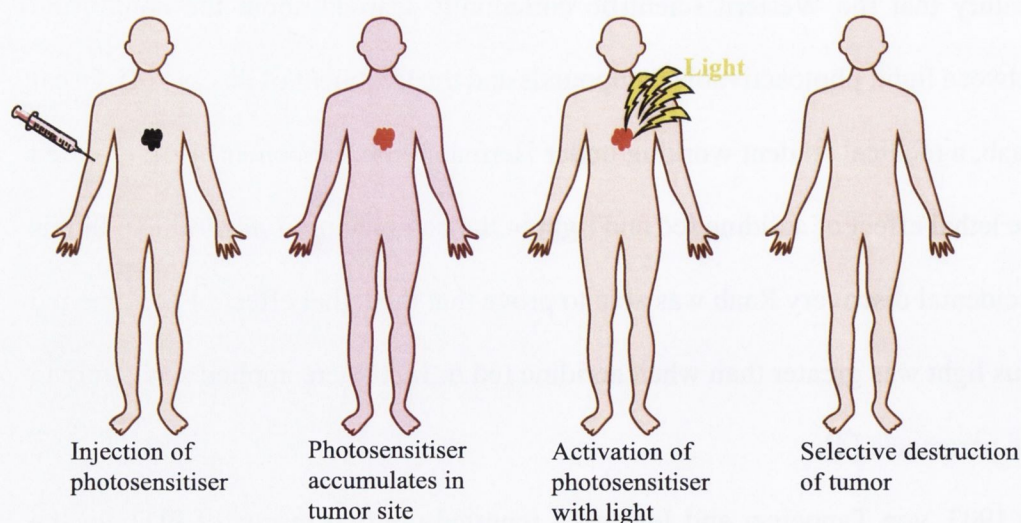


Figure 1.1.1 Basic principles of photodynamic therapy in patients bearing solid oesophageal tumours.

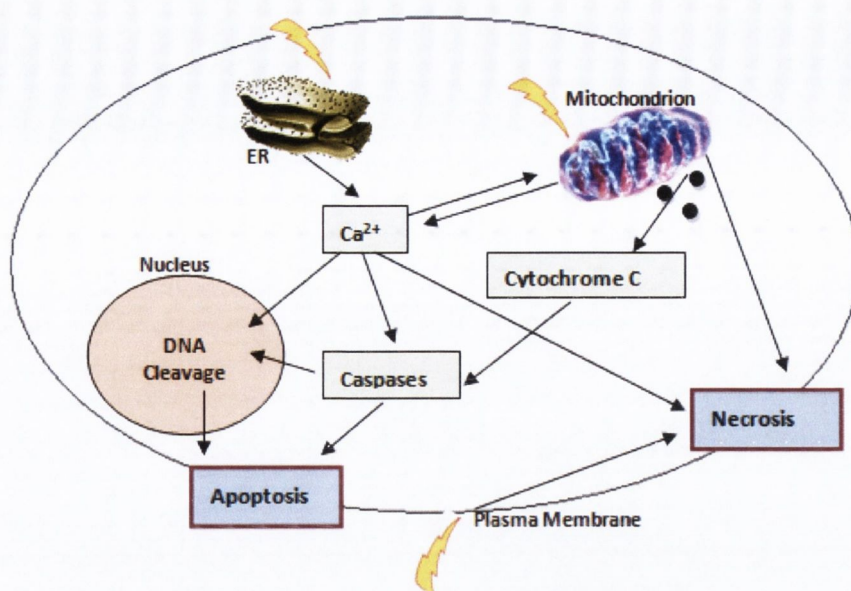


Fig. 1.1.2 Major intracellular cellular events that occur during and after PDT.

At a molecular level, there are two mechanisms responsible for cell death during PDT [11]:

- Type I reactions (Fig. 1.1.3 and 1.1.4) “are characterized by a dependence on the target – substrate concentration” [12]. These occur when the excited PS transfers an electron directly to an organic substrate resulting in an oxidised substrate and reduced PS, it can then react with superoxide radicals to produce superoxide anions which can then create the highly reactive hydroxyl radical (Fig. 1.1.4) [11, 13]. These reactions are most common if the photoreaction is occurring in a hypoxic environment [12, 13] and all lead to the destruction of cells.
- Type II reactions (Fig. 1.1.3 and 1.1.4) “are characterized by a dependence on oxygen concentration” [12]. Even though these types of reactions are most commonly associated with the production of $^1\text{O}_2$, other compounds in the triplet state may be involved in the photoreaction (Fig. 1.1.2 and 1.3.4) [11, 13]. Nitric oxide and vitamin A are just two

examples [12]. Both these mechanisms cause oxidative damage to key molecules within the cell leading to irreversible cellular damage ultimately resulting in cell death by necrosis and/or apoptosis (Fig. 1.1.2).

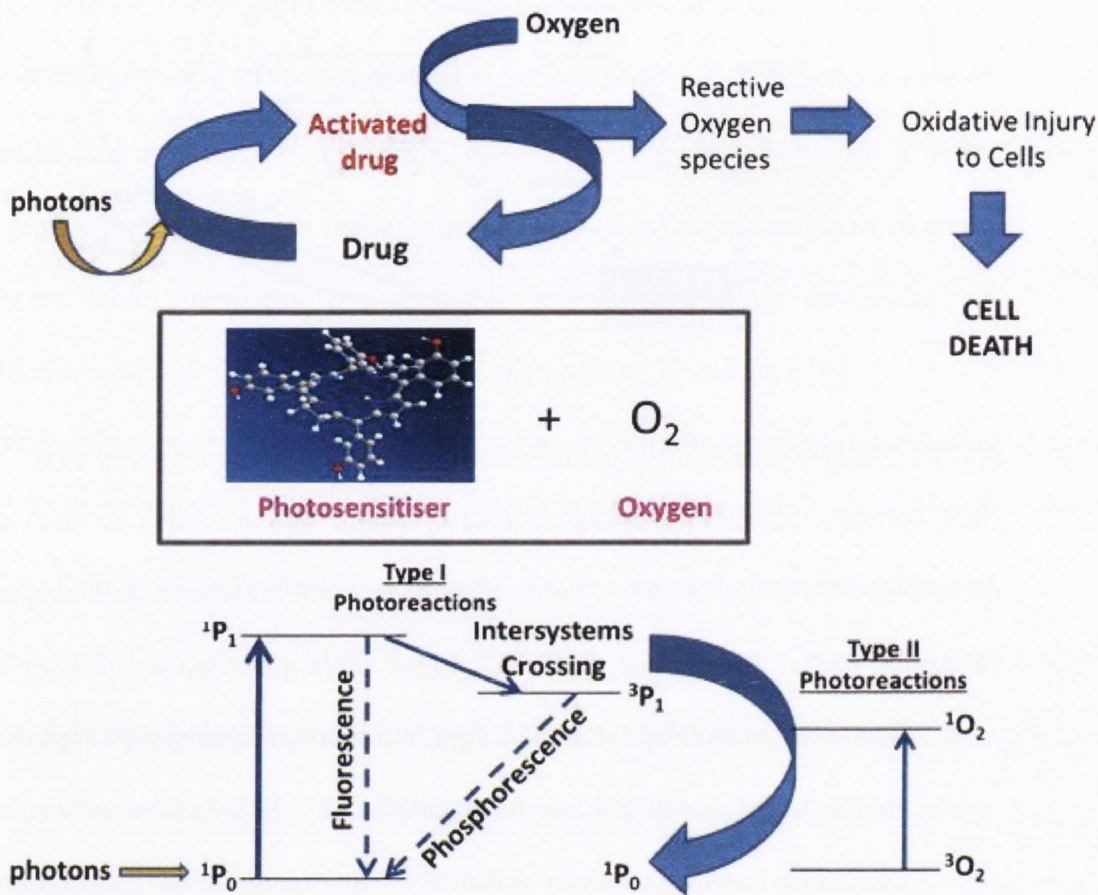


Figure 1.1.3 Graphic representation of the macromolecular and molecular mode of action of PDT.

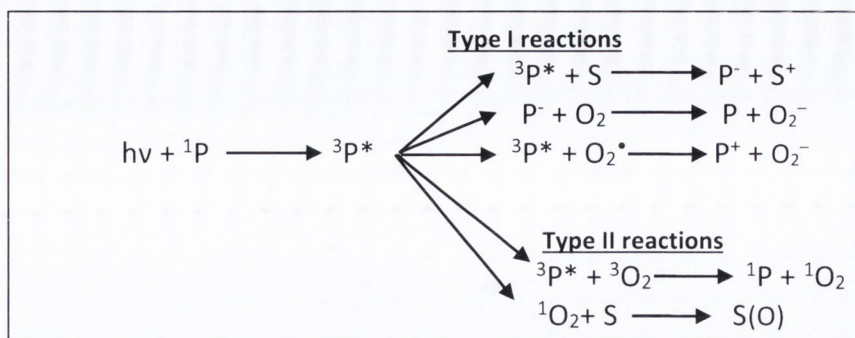


Figure 1.1.4 Different types of reactions that may occur during treatment with PDT [12]. ${}^1P^-$ – photosensitiser in singlet ground state; S – substrate; ${}^3P^*$ – photosensitiser in triplet excited state; P^- – reduced photosensitiser; S^+ – oxidized substrate; O_2 – molecular oxygen; O_2^- – superoxide anion; $O_2^{\bullet -}$ – superoxide radicals; P^+ – oxidized photosensitiser; 3O_2 – triplet ground state oxygen; 1O_2 – singlet excited state oxygen; $S(O)$ – oxygen adduct of a substrate.

Singlet oxygen (highly reactive species) is the main cytotoxic agent produced during PDT [12]. When the oxygen concentration is reduced from 5% (the concentration in normal tissue) to 1%, the PDT effect is halved [11]. This suggests that type II reactions must be predominant [12] during PDT treatments; however, type I photoreactions still occur [1]. The relative contributions of these in real biological systems strongly depend on the concentration of molecular oxygen and the concentration and reactivity of other oxidizing compounds, among many other factors; in fact, it is very likely that the contributions of type I and II photoreactions can change during the time of the photochemical process [1].

The short lifetime of singlet oxygen (reduced from 3 μ s in water to approximately 100 ns in biological environments [14, 15]) explains why PDT damage occurs close to cell and tissue regions of high photosensitiser concentrations and why PDT has a low genotoxic potential [11].

The success of PDT in cancer treatment relies on the selective uptake of the PS by tumour tissue [16] due, primarily, to the increased permeability of the tumour

vasculature [16] and a higher retention of the drug by the tumour tissue due to its increased proliferative capacities and altered cellular metabolism [16].

PDT is a local rather than systemic treatment [17] so it is suitable only for localized solid tumours, following the application of the photosensitiser (Fig. 1.1.1), it is necessary to wait for the PS to localize in the tumour tissue and this area is subsequently irradiated (Fig. 1.1.1), leading to a localized photoreaction that results in the destruction of tumours [17]. There are many other manners of delivering PS agents to sites of tumour growth, including, inserting PS drugs in specific nano-carriers such as targeted liposomal structures [18, 19] and polymeric pro-drug delivery platforms that can be designed to activate only when having reached the tumour itself [20].

Even though singlet oxygen is highly reactive has a rather low diffusion (0.2 μm) [17] hence tissue damage is severely limited by the depth of penetration of the light used during treatment. However, PDT also severely damages tumours vasculature leading to further destruction of the tumour by depriving the tumoral tissue of oxygen and nutrients as well as eliciting a crucial immune response (Fig. 1.1.5) [17, 21]. This anti-angiogenic facet of PDT translates into a higher efficacy of treatment of solid tumours as it provides a parallel way of inducing tumour destruction (Fig. 1.1.5).

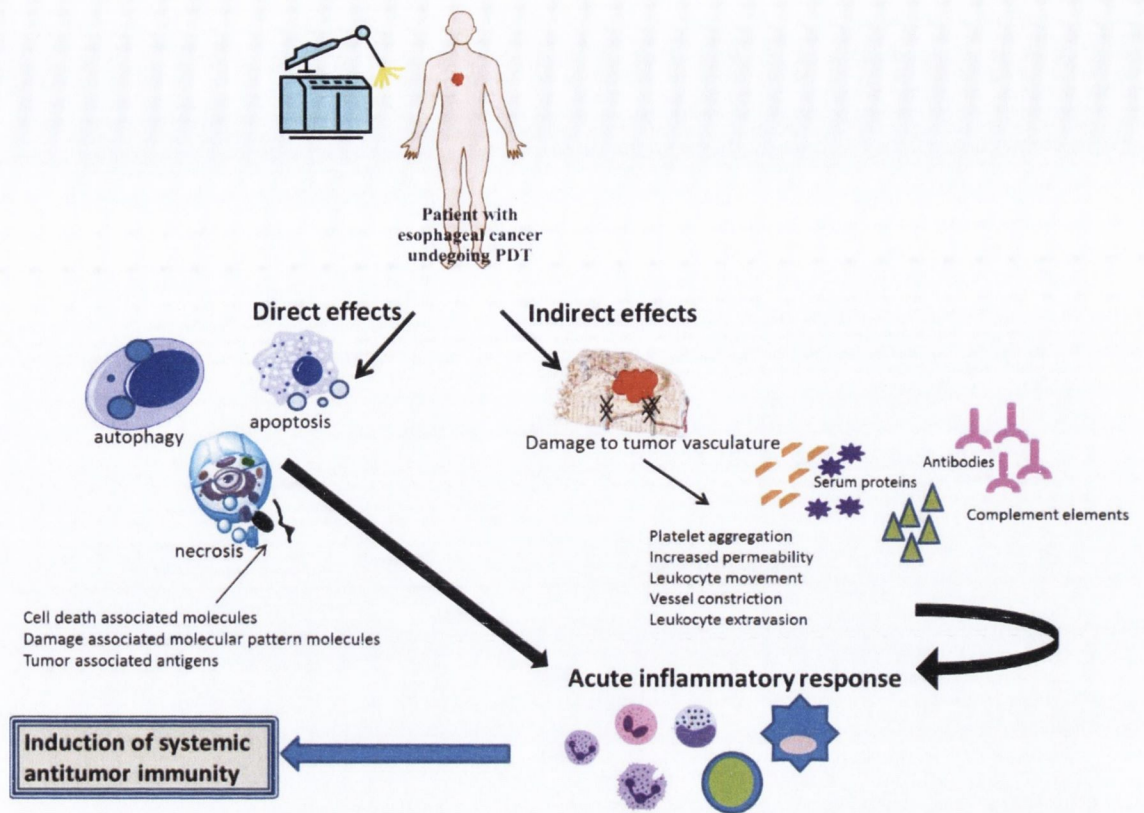


Figure 1.1.5 Schematic representation of the main mechanisms of cell death and inflammatory effects following PDT.

Unlike most of the anti-cancer treatments PDT treatment it is known to result in a mixture of apoptotic and necrotic cells [22] (Table 1.1.1). In fact it is thought that the resulting necrotic cells leaking their highly antigenic contents (for example heat shock proteins) onto adjacent intracellular spaces elicit an immune response critical for the successful treatment of tumours using this therapy [22].

Table 1.1.1 Major cell death mechanism activated by PDT, affected organelles and major biochemical events.

Antitumor PDT mechanisms	Organelles	Processes
Direct Cell Damage	Mitochondria * Release of Cytochrome C * Bcl-2 damage	Apoptosis
	Cytoplasm * NFkB damage	
	Endoplasmatic reticulum * Beclin 1 * mTOR activation	Autophagy
	Cell membrane disintegration	Necrosis
Vascular damage	Local depletion of oxygen and nutrients	Apoptosis Necrosis Autophagy
Activation of immune response	Cytotoxic T cells	Granzyme mediated apoptosis

The most common immune response to PDT is thought to be mediated by an acute inflammatory response responsible for the complete destruction of the tumour (Fig. 1.1.5) [23].

A large infiltration of leukocytes to the PDT treated site caused by the release of immune stimuli is followed by an organised response by the immune system causing further tumour destruction [24]. In fact, it is the later that actively

identifies and eliminates tumour cells even in remote locations. This immune action is thought to be responsible for long term tumour control [24, 25]; here it is known that hours after the PDT treatment an acute immune response is responsible for further tumour destruction, however, a subsequent adaptive immunity provides a long term, sustainable immune commitment against tumour regrowth [24, 25]. Animal experiments suggest that mice with compromised immune systems tend to have a reoccurrence of the tumours while mice with healthy immune systems remains tumour free following PDT treatment [22].

Unlike traditional therapies (surgery and chemotherapy), also spares adjacent healthy tissues; it can also be administered in an outpatient setting (as it is minimally invasive [26]), allowing the patient to undergo re-treatment with no cumulative toxicity. There is also no evidence that tumours become resistant to this course of treatment meaning that multiple interventions with PDT would lead to similar results to those obtained in previous treatments [17].

PDT offers many advantages over traditional cancer treatments; however, the most significant are: low mutagenic potential, low system toxicity and tumour destruction is more selective [1, 9].

PDT today remains a novel form of treatment with many side effects. The currently used photosensitisers still have prolonged leaving the patient light sensitive for many weeks following injection and even illumination.

1.2 PDT and oesophageal cancer

Oesophageal cancer is the second leading cause of death by cancer in Ireland [27, 28] with an incidence of 8.7% in 2012 [29]. A special point of interest is Barrett's oesophagus, the precursor lesion to oesophageal adenocarcinoma [30]. Barrett's oesophagus is a complication of gastroesophageal reflux disease

(GERD) [31] where the continuous and prolonged action of refluxed stomach acid and chronic inflammation [32] turn the normal squamous epithelium into a pre-malignant metaplastic columnar epithelium [33]. It is characterised by widespread and continuous epigenetic methylation especially in tumour suppressor genes [34]. However, only 5-10% of patients with GERD go on to develop full adenocarcinoma meaning that genetic makeup plays a central role in the development of oesophageal adenocarcinoma [34].

Nonetheless, it is a well-known fact that pre-existent conditions such as Barrett's oesophagus severely increases the risk of development of oesophageal adenocarcinoma [35]; has been estimated that Barrett's sufferers have a 30 to 125 fold increased chance of developing oesophageal malignancies [34].

Oesophageal cancer is a malignancy of the oesophagus that is clinically characterised by dysphagia and weight loss [36] it is also considered a highly deadly illness mainly due to the fact that diagnosis usually occurs late into the progress of the disease [36, 37] with a mortality of 7.2% of patients in Ireland [29]; 5 year survival rate remains between 15% to 25% [36, 38]. It is also thought to have one of the highest malignancy potential of all tumours [39]. The mortality is so high that oesophageal cancer is the 7th source of cancer related deaths worldwide [37, 40].

Before the 1970's this malignancy was considered rare, however, an exponential increase in its incidence coincided with changes in lifestyle and diet of westernised populations with high intake of dietary fats, smoking, drinking and a low exercise regime [41, 42]. Overweight middle age white males with high dietary fat and alcohol intake allied to a low exercise regime are at the top of the risk factors for this disease [41].

There are two main types of oesophageal cancer:

Oesophageal squamous cell carcinoma:

- the most common of the two types of cancer [36]
- characterised by a rapid proliferation of squamous cells in the upper oesophagus [36]

Oesophageal adenocarcinoma (Barrett's metaplasia):

- incidence on the rise in the UK, Australia, USA and Ireland [28, 36]; the incidence of adenocarcinoma has surpassed that of squamous cell carcinoma in the USA [36] and in Ireland [28]
- characterised by a rapid proliferation of highly secretory gland cells in the lower oesophagus [43].

Although surgery (oesophagectomy) is currently considered to be the “gold standard” [38] such interventions are invasive, expensive; they involve a hard and long recovery period; also many advanced cases of oesophageal cancer are not always eligible to undergo such therapies [44].

PDT represents a potential step in the treatment of these and related cancers and is currently only used as palliative treatment to reduce dysphagia [37]. A recent study in a cohort of United Kingdom patients suggests that patients that had undergone palliative treatment with Temoporfin-PDT for head and neck carcinomas showed an excellent response when compared to equivalent palliative chemotherapy or surgery; but also, surprisingly, 16% showed remission of the cancer *in situ* unlike traditional palliative treatments [10]. In addition, Temoporfin-PDT was found to be more cost-effective than surgery and chemotherapy [10]. It is known that patients with head and neck malignancies often develop tumours lower in the gastro intestinal track [45, 46], hence this

study could have a direct correlation on the treatment of primary oesophageal malignancies.

1.3 Photosensitisers

In PDT the irradiation of a photosensitizer sets off chemical reactions that produce transient cytotoxic compounds [47]. In theory, any molecule that goes into a triplet state, when absorbing light at a specific wavelength and able to transfer its energy to ground state O_2 , could be used in PDT. However, several factors limit the number and types of molecules that can be used as PS. Namely, toxicity of compounds, specificity for unhealthy tissues, retention of the drug in the desired tissues, rate of photobleaching [47], just to name a few.

Ideally, a PS should be non-toxic, water soluble, with a high quantum yield of singlet oxygen, selectively retained, in high concentrations, in the target tissue and readily cleared from the body (mainly the skin) in order to avoid continued photosensitivity [12].

Light penetration is weak in living tissue and tends to be dispersed by endogenous chemical species like melanin, haemoglobin and water [11], so the therapeutic window of light in living tissue lies between 600 nm and 1300 nm. This means that any PS should absorb at the highest possible wavelength in the light spectrum (i.e. red/infrared) [11]. On the other hand, PS have to have the ability to undergo an intersystem crossing to a triplet state [48] and photons above the 830 nm barrier do not carry enough energy for this conversion to occur [11]. An ideal PS should have a high extinction coefficient in the 600–800 nm range, where light penetration into tissue is maximal, and where the photons still have enough energy to lead to the formation of 1O_2 [49].

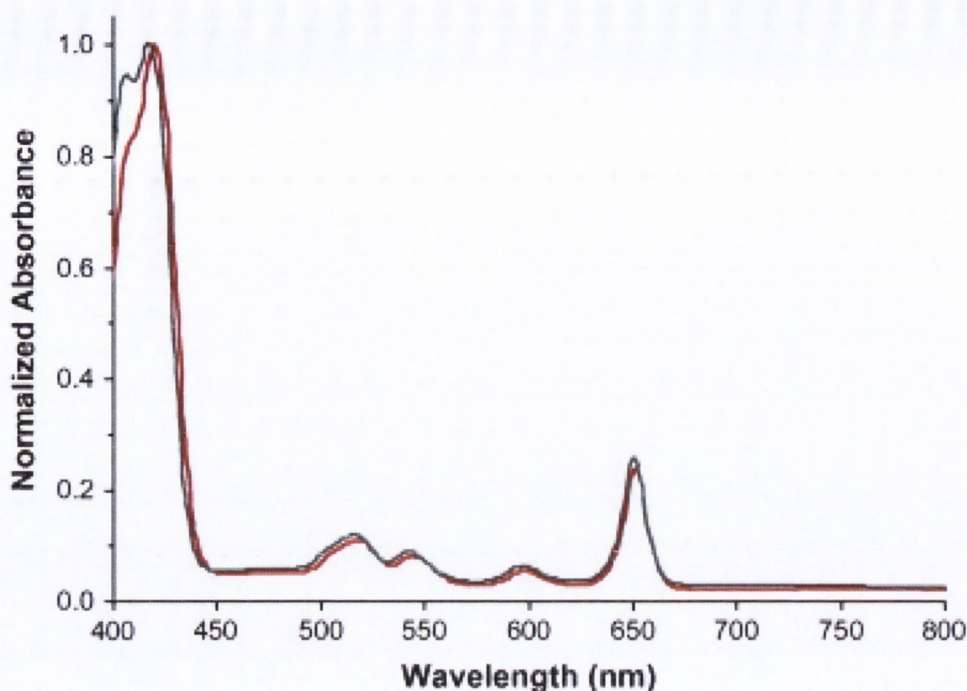


Figure 1.3.1 Graphic representation of the absorption profile of Temoporfin (red) in methanol [50]. Temoporfin is a clinically approved second generation porphyrin photosensitizer.

Most commonly, photosensitizers are porphyrin derivatives due to, not only, their high absorption in the red - near infrared region of the light spectrum (Fig. 1.3.1) but also they are naturally occurring compounds that can be chemically synthesised and modified using “simple” chemical synthetic approaches.

In the 1970’s when hematoporphyrin (Hp) was used as a photosensitiser drug, the need to purify Hp led to the addition of sulphuric and acetic acid during a purification step [11]. This led to the formation of hematoporphyrin derivative (HpD) [11] which is still used today as a PS (Table 1.3.1). These first generation photosensitisers were crude mixtures of compounds that exhibited several drawbacks such as a low absorbance at the near infrared region, prolonged photosensitivity, low purity and prolonged photosensitivity to patients [11, 49].

This led to the development of second and third generation photosensitisers [11, 49].

Second generation PS include porphyrins, phthalocyanines, naphthalocyanines, benzoporphyrins, bacteriochlorins, chlorins, just to mention a few [11, 49]. These compounds can be obtained through chemical synthetic methods yielding chemically pure drugs that absorb between 650 to 800 nm in the light spectrum (these are ideal wavelengths in terms of tissue penetration) [49]. They are very effective generators of singlet oxygen and reduce time of patient photosensitivity [49]. Both 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (*m*THPC – Temoporfin) and benzoporphyrin derivative monoacid (BPD-MA) Visudyne are second generation photosensitisers and have been given regulatory approval for clinical use (Table 1.2) [11].

Second generation photosensitisers coupled to carrier systems are now being evaluated as potential “third generation” photosensitisers, these are thought to accumulate more selectively in tumours [49], reducing even more the time for which the patient presents photosensitivity and could potentially reduce the amount of drug given to the patient. Novel carrier systems such as monoclonal antibodies, liposomes, antibody fragments and nanoparticles are now under study [11].

Table 1.2 Photosensitisers for photodynamic therapy, diseases treated and their status of approval [1, 17, 51-65]

PS	Commercial name	Indication	Activation wavelength (nm)
Drugs with regulatory approval			
<i>m</i> THPC	Temoporfin	Head and neck cancers	652
BPD-MA	Verteporfin	Age-related macular degeneration	689
5-ALA	Ameluz	Actinic keratosis	635
5-ALA-methyl ester	Metvix	Actinic keratosis; basal cell carcinoma	635
HpD	Photofrin	Oesophageal dysplasia and early cancers	630
Drugs under clinical development			
BPD-MA	Verteporphin	Basal cell carcinoma (BCC)	689
<i>m</i> THPC	Temoporfin	BCC; prostate and pancreatic cancer	652
HpD	Photofrin	Brain tumours	630
SnEt2	Purlytin	Cutaneous metastatic breast cancer; BCC; Kaposi's sarcoma; prostate cancer	664
Lutetium texaphyrin	Lutex	Cervical, prostate and brain cancers	732
Phthalocyanide-4	Pc4	Various cancers	670
AIPcS	Photosan	Head and neck cancers	670
Taporphin sodium	Talaporfin	Various solid tumours	664
Pd-Bacteriopheophorbide	TOOKAD	Prostate cancer	763
5-ALA-methyl ester	Hervix	Diagnosis of bladder cancer	~ 400

1.4 Light sources

The first light sources used for PDT were conventional lamps that produced a high amount of heat and did not generate coherent light; coloured filters were used to select narrower wavelengths [6].

In December 1995, the Food and Drug Administration (FDA) approved Photofrin (porfimer sodium) [66] for treatment of oesophageal cancer in the United States. The 630 nm light used to activate the PS was to be delivered by fibre optics coupled to a 630 nm emitting laser [26]. In Europe the European Medicines Agency (EMA) approved the use of Temoporfin (*m*THPC) in October 2001 for the treatment of advanced head and neck carcinomas [67]. This PS is activated using fibre optics coupled to a 652 nm emitting laser [26]. One of the facts that prevent the widespread use of PDT is the historical lack of advances in visible light delivery technology and respective emitting devices [26]. Today technology has advanced to a point where light delivery devices are small and have been simplified [26]. However, the fact that most light sources for PDT are laser-based means that health professionals involved in these types of therapy must be formally trained not only in the safe use of laser light but it also involves training in proper dosimetric delivery of light [26]. It also means that the light sources remain expensive and impractical [26].

The choice of light source depends on the desired depth of tissue penetration [6, 68], the location of the tumour, the light dose to be delivered and the type of photosensitiser to be activated [47]. An important factor to take into account when choosing an appropriate light delivery system is the optical penetration depth (OPD), this is defined as the depth at which light attenuate 37% of its initial value [47]. For example, at 630 nm, brain tissue has an OPD 0.8 mm while bladder tissue has an OPD of 4mm at the same wavelength [47].

The depth of penetration of light increases near the red and infrared region of the light spectrum [6], however, the specific wavelength used when treating a patient has to be chosen according to the maximum absorbance of the administered PS in order for production of a maximal yield of singlet oxygen [6]. There are only a handful of approved light delivery systems for PDT and these are summarized in Table 1.4.1.

The most common used light sources in clinical PDT are lasers not only because these can be used to deliver monochromatic light but also because this type of light can be easily channelled through specialized delivery systems such as optical fibre cables and other adapted delivery devices depending on the site to be illuminated [69, 70].

Laser systems are large and expensive but they are increasingly being replaced by new generation laser systems (for example diode lasers) and even non-laser systems which are much smaller, cheaper and safer [26, 47].

Non-laser light sources (such as LED systems) counteract the need to use expensive equipment, few security features are necessary, there is no need to couple to a fiberoptics system allowing a larger area to be illuminated in a single application and no specific training has to be provided to health professionals [47]. These novel systems not only result in an equivalent effect to that of a laser system but can also be used with a wider spectrum of PS [5].

In photodynamic therapy, the light delivered to the lesion site is characterised by both dose (the energy of the light applied to a certain area) and fluence rate (the power of the light applied to a certain area) [71]; these have a profound influence on the effectiveness of PDT [72]. This influence is such that the concept of PDT dose had to be introduced; this is defined as the number of photons, per gram of

tissue, absorbed by the photosensitiser taking into account the photobleaching of the drug during treatment [70].

In clinical practise it is widely accepted that for an optimum PDT effect light delivery must be of low fluence and high dose as this allows for oxygen levels in the target tissue to readily recover [21, 73].

Clinical protocols of illumination vary according to the type of condition to be treated, the photosensitiser used and the target area (size of lesion, cancer, etc and location in the body). For example, *m*THPC can be administered to patients bearing superficial oesophageal tumours and tumour site is then illuminated with green light (dose 100 J/cm²) with little to no side effects (such as fistulas or stenosis), the use of windowed balloons may also reduce oesophageal stricture [37, 74, 75]. Some recent studies also suggest that a pulsed/fractioned light delivery would increase the effectiveness of PDT [68, 76-78].

Table 1.4.1 Commercially available laser systems available for PDT, their spectral characteristics and associated delivery methods.

	Wavelength (s) nm	Bandwidth	Fluence rate	Pulse duration	Light delivery
Argon laser	488 and 514.5	Monochromatic	0.5-1 W/cm ²	Continuous wave (CW)	Direct /optical fibre
Dye laser pumped by argon laser	500-700 (dye dependent)	5-10 nm	10-200 mW/cm ²	CW	Direct /optical fibre
Metal vapour laser	UV or visible (metal dependent)	Monochromatic	Up to 10 W/cm ²	10ps- 50ns quasi-cw	Direct /optical fibre
Dye laser pumped by metal vapour laser	500-750 (dye dependant)	5-10 nm	10-500 mW/cm ²	10ps- 50ns quasi-cw	Direct /optical fibre
Solid state laser	1064, 532, 355, 266 (for Nd:Yag)	Monochromatic	Up to 10 W/cm ²	10ps- 30ns quasi-cw	Direct /optical fibre
Dye laser pumped by solid state laser	400-750 (dye dependent)	5-10 nm	10-500 mW/cm ²	10ps- 30ns quasi-cw	Direct /optical fibre
Solid state optical parametric oscillator	250-2000	Monochromatic	Up to 1 W/cm ²	10ps- 30ns	Direct /optical fibre
Semiconductor diode lasers	600-950	Monochromatic	Up to 700 mW/cm ²	CW	Optical fibre

1.5 High content technology

High Content Analysis (HCA) is a convergence between cell based assays, high resolution fluorescent imaging, automation and advanced image analysis by analysis software [79]. It has been widely adopted by pharmaceutical industry and some academic centres for the validation of potential drugs [80] and to elucidate the mechanisms of drug action at a cellular and subcellular level.

It is a powerful method that allows the simultaneous detection of multiple biological pathways, the pre-clinical toxicological evaluation of drugs and can even be used (when coupled to an automated system) to screen for the effects of thousands of potential drugs within a relatively short amount of time. This experimental methodology is denominated high content screening (HCS) [80]. It is particularly valuable during early drug development, and provides a large-scale biological platform with significant potential for automation and miniaturization [81]. HCS links various independent fields from synthetic chemistry to biology through toxicology (Fig. 1.5.1) and from computational sciences to advance microscopy [82]. HCS has received much interest in the last decade hence new “rules” for the use and development of relevant assays have been established (Fig. 1.5.1).

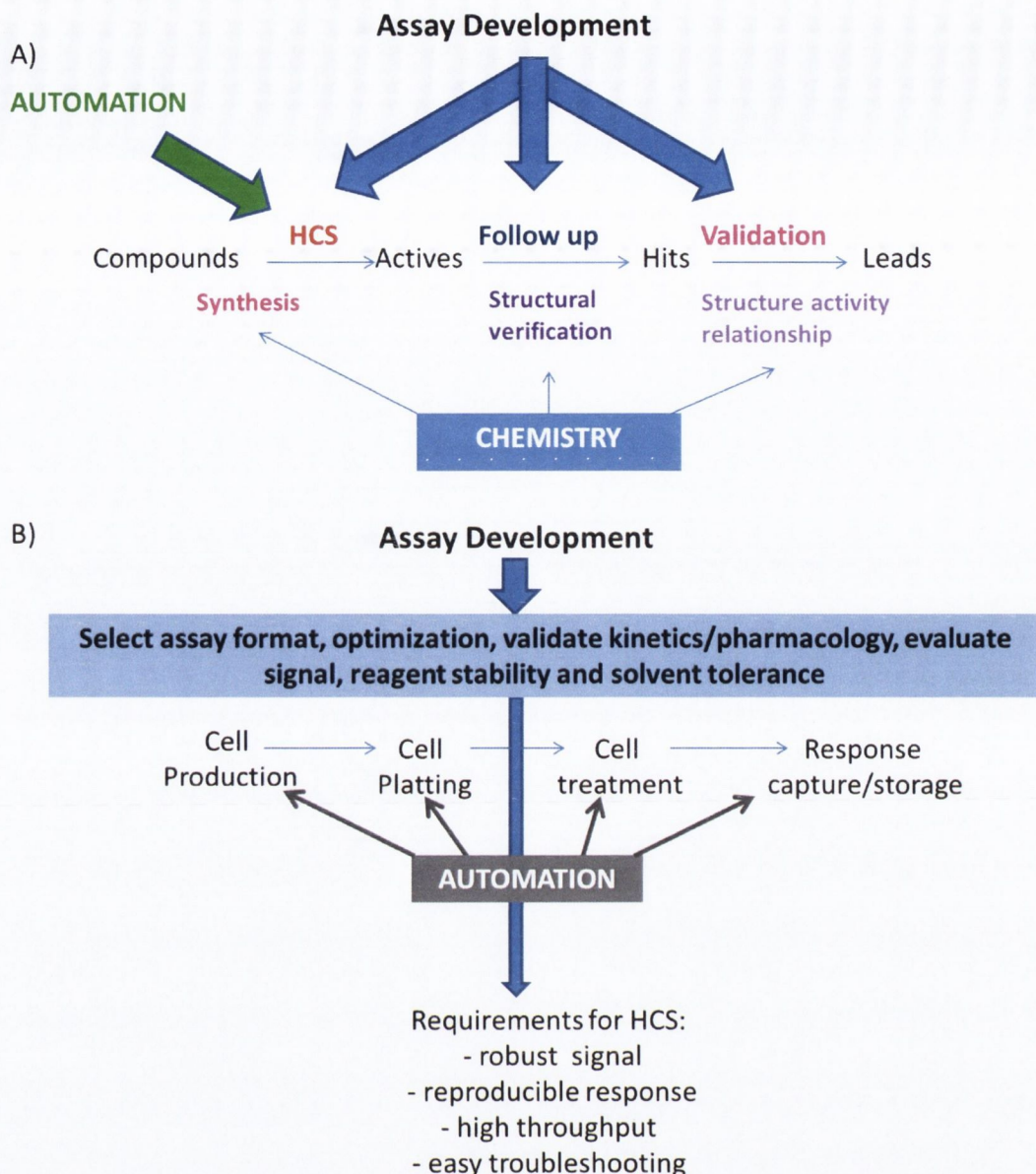


Figure 1.5.1 Summary of the two main strategies in the development of a HCS assay using potential toxic compounds. **(a)** “Lead generation. A lead generation strategy must be developed for primary and secondary assays for the confirmation of HCS actives, hit assessment, lead validation and lead optimization. **(b)** Cell-based assay development. Additional challenges to the development and implementation of cell-based screens beyond those associated with biochemical HCS formats” [82].

HCS is based on the use of fluorescent labelled cells that are photographed using automated microscopes [83]. These images are then analysed using automated image analysis software allowing the quantification of several phenotypic characteristics of the assay cells [83]; this technique generates an enormous

amount of data that needs to be filtered through in order to obtain biologically relevant information [83]. In order to overcome this obstacle, data mining techniques have been developed linking biology to computer technologies.

HCS is not only a robust technique but also very versatile as many types of assays can be performed using the same platform, from toxicology to validation of targets, to RNAi experiments. The assay scientists also have available a broad range of cellular platforms (Table 1.5.1) from microscopy slides to 394 well plates.

HCS owes its generalised acceptance and progress not only to its versatility but also to the fact phenotypic screens have yielded many commercially successful drugs in the past decade [84, 85]. More powerful tools in the field of automated fluorescent imaging and a strong commercial interest from leading pharmaceutical companies also allowed for the rapid dissemination of HCS worldwide. They also sparked major advances on the technological side with ever more powerful hardware (Table 1.5.1) as well as an ever increasing array of commercially available fluorophores [86] such as Hoechst (a bis-benzidine blue fluorescent dye used to stain DNA) [87].

Table 1.5.1 List of commercially available HCS platforms, associated optics and image analysis software offered.

Company	Brand name	Light source	Optics	Image analysis software
Amnis	ImageStream MarkII	Laser/Lamp	Wide field	INSPIRE
BD Biosciences	BD Pathway 855	Lamp	Wide field/Confocal	BD Pathway software suite
	BD Pathway 435	Lamp	Wide field/Confocal	BD Pathway software suite
GE Healthcare	IN Cell Analyzer 2200	Lamp	Wide field	In Cell Investigator
	IN Cell Analyzer 6000	Laser	Wide field/Confocal	
Intelligent Imaging Innovations	3i VIVO	Lamp	Wide field/Confocal	SlideBook™ software.
	3i VIVO 2 Photon	Lamp	Wide field/Confocal	
	3i EVEREST	Lamp	Widefield	
	3i Marianas	Lamp	Wide field	
Leica Microsystems	TCS SP5	Laser	Confocal	Leica HCS A software
Molecular Devices	ImageXpressULTRA	Laser	Confocal	MetaXpress
	ImageXpress® Velos	Laser	Wide field	
	ImageXpressMICRO	Lamp	Wide field	
Olympus	Scan^R	Lamp	Wide field	Scan^R scan analyser
Perkin Elmer	Opera	Laser	Confocal	Acapella
	Operetta	Lamp	Wide field/Confocal	
Thermo Scientific	Cellomics ArrayScan XTI	Lamp	Wide field/Confocal	BioApplications
	ArrayScan™ Infinity High Content Reader	Lamp	Wide field/Confocal	
	CellInsight™ NXT	Lamp	Wide field/Confocal	
TTP LabTech	Acumen eX3	Laser	Wide field	-
Roche	Cellavista	Lamp	Wide field	Cellavista Software Workstation

HCS in the field of toxicology has mainly been applied in the prediction of hepatic toxicity of large libraries of compounds [88, 89]; however, novel phenotypic based toxicity screens have been increasingly used by pharma and academia alike [85, 90, 91].

The study of the *in vitro* interactions of any naturally fluorescent molecule with cells, such as porphyrins and their derivatives, poses many difficulties. In the field of PDT assessment and development the most prominent are:

- when working with photoactive compounds, most experimental manipulations of living cellular material need to be carried out under special illumination conditions (natural light activates these compounds and prematurely produce cellular damage)
- the fact that these compounds have been designed to convert light energy into cytotoxic free radicals translates into easy photodegradation in the presence of any light [92, 93]
- by their very nature these compounds have an intrinsic fluorescence over a broad range of excitation and emission wavelengths [94]. This turns analysis of these chemicals with techniques based on fluorescence analysis with various chromophores challenging.

Traditionally a large number of methodologies are used for the drug evaluation, among these are:

MTT assay - This is an indirect method widely used to measure cell health, viability and growth. It is based on the principle that viable cells are able to metabolically reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into its insoluble form formazan inducing a colour change (from yellow

to purple); results are read as a measurement of absorbance [95-99]. Though a much used technique in the evaluation of the effect of various toxicants in cells, indirectly measuring toxicant efficacy, it does have some shortcomings. For example, it needs an incubation period prior to a stop and solubilisation step before reading which not only destroys the cells, adding unnecessary time which is detrimental to high throughput screens, but also makes it impossible to further analyse the cells. It also offers a single readout and for other parameters such as drug uptake further experiments are needed. MTS is equivalent to MTT but it does not require the solubilisation step [100].

BrdU - Indirectly measures drug efficacy; it is based on the principle that 5-bromo-2'-deoxyuridine will be incorporated into genomic DNA during synthesis in dividing cells. An anti-BrdU antibody is then added and absorbance is detected [98, 101]. This technique, though effective, has its drawbacks, it uses an antibody which often needs optimization; it is expensive and adds several steps to the process. It also involves a denaturing step which could negatively affect the cells in study. Again, this is a single output assay.

Flow cytometry - This widely used technique is based on the evaluation of fluorescent labelled cells, previously stained with dyes that elucidate biologically relevant events. It can be used to determine events such as toxicity [102], apoptotic events [103] and DNA content among many other parameters. There are, however, downsides to this technique, in adherent cell lines trypsinization needs to be performed before use, this can cause cell damage and/or inadvertent uptake of the compounds. It also adds an extra step; cellular response and uptake in the same cells cannot be compared nor related. In addition retrieval of cells post analysis is improbable.

Trypan blue exclusion - This method is based on the premise that living cells are able to actively exclude trypan blue from the cytoplasm, while dead or dying cells are permeable to the dye. Results are read in a microscope by a technician or an automated cell counter. Though very effective, the human element may induce error. It is difficult to quantify and must be performed in live cells [104, 105]. The time needed to count the cells may lead to cell death not related to the action of the tested drugs. Automation of this technique is difficult and, in the case of adherent cells, involves a trypsinization step that involves more manual handling of the cells and may induce further error in the results and loss of cells due to processing rather than drug action.

Neutral comet assay - This electrophoresis assay detects the fragmentation of DNA resulting from drug action [106]. It involves the lysis of whole cells which is time consuming and although informative of apoptosis in a population of cells would be hard to automate and unsuitable for a high throughput primary screen.

Neutral red assay – assessing cell proliferation through the incorporation of neutral red by viable cells, the dye is then extracted and absorbance read at 540 nm [99]. Though simple in nature this assay still has many limitations such as the limited potential for automation, errors related to drugs that affect lysosomal function (may render false positives), and the fact that the dye must be extracted from the lysosomes would add an unnecessary work load. With these issues and its single output result, this technique would not be suitable for a large high throughput primary screen. [107, 108]

Raman – This vibrational spectroscopy can be used to detect toxicity by evaluating known molecules involved in toxicological reactions in cells, such as cytochrome c that is released from mitochondria during cellular photodamage

[99]. This method is manual and lengthy with need for a highly skilled and trained operator; it does not give any information on the drug itself, hence changes detected may not be the result of drug action. It is not automatable hence it would be unsuitable for a large primary screen.

Confocal imaging – Based on the optical evaluation of cells, this is a technique can be used to determine the localization of fluorescent compounds and dyes in the cell and even specific subcellular compartments in conjunction with known, commercial dyes, to show localization [109] and uptake [110]. It is mostly manual, with little possibility of automation and only evaluates, at most, a few cells at a time. Though useful this technique is difficult to integrate a high throughput primary screen not only due to the manual nature of the assay but also the limited image analysis potential [109-111].

HCA has many advantages over the more traditional approaches. For example, it can be used as a one-step assay to screen a library of PDT compounds as its output may include parameters related to the effect of the PS on cells but also offers parameters related to the intracellular presence of the drug itself. However, all of the above mentioned techniques pose issues when it comes to screening large libraries of compounds (Table 1.5.2).

Table 1.5.2 Summary of various methods traditionally used for determining efficacy of compounds in *in vitro* tests

Method	Suitable for a large primary screen	Why
HCA	√	high throughput, automatable, fast, multiple outputs allows for spatial distribution of macro molecules
MTT	×	overnight incubation needed before reading, single output, destructive
BrdU	×	needs antibody, requires denaturation step, lengthy
Flow cytometry	×	needs trypsinization, optimization needed, no drug parameters
Trypan blue exclusion	×	manual, lengthy, automation not viable
Neutral comet assay	×	needs lysis of cells, single output, no drug parameters
Neutral red assay	×	read at 540nm (maximum absorbance of our porphyrins)
Raman spectroscopy	×	labour intensive, single output, low throughput
Confocal imaging	×	low throughput, not automatable

When determining toxicity. HCA is an ideal assay to screen a library of PDT compounds as its output may include parameters that relate directly to the potential PS (due to their inherent fluorescence) in the cells such as intensity and area occupied. It also allows for the study of live cells and results are obtained in intact cells [81, 112]. The use of HCA techniques in a screening setting has evolved into its own field, high content screening (HCS) [80, 81, 112]. This technique allows for the simultaneous detection of multiple biological pathways and the pre-clinical toxicological evaluation of pharmaceutical drugs. *In vitro* estimation of toxicity using HCS in cell lines has been used in recent years, particularly in predicting hepatic toxicity [88], but also to assess toxicity of anticancer agents [113].

Both HCA and HCS are, in essence, very simple methods [80]. Based on *in vitro* evaluation of potentially therapeutic compounds [80], they enable simultaneous measurement of multiple features of cellular phenotype that are relevant to therapeutic and toxic activities of those compounds.

This work aims to setup and validate a semi-automated high content screening method for the assessment of toxicity of photodynamic porphyrins as possible PDT agents.

1.6 3D tumour models and their relevance in the field of development of new therapeutics

Solid tumours are complex tissues that are not easily grown in a laboratory setting; however, and greatly due to the advances in commercially available and easy-to-use technologies (Table 1.6.1), spheroids and other tumour mimics are increasingly being used in *in vitro* analyses. These tissue analogues can be analysed by a variety of technologies from MTT to high content imaging and analysis.

Most *in vitro* tissue evaluations are done in either cell monolayers or, if the cells are non-adherent, tests are performed in cell suspensions. However, the fact remains that cells in a three dimensional (3D) architecture behave fundamentally differently to cells cultured in monolayers [114]. Laboratory evaluations have as a central principle that cell culture conditions and behaviours reflect those in whole organisms, organs or tumours [115]. Yet, it is imperative, particularly in tumour biology, that the tumoural microenvironment generated in the presence of solid three dimensional structures is replicated in a laboratory setting; hence,

the generation of 3D tumour like structures *in vitro* has been a prolific field [114-118].

The basic principle behind the generation of 3D cell cultures is that if cells naturally grow in 3D structures, simulating this type of architecture *in vitro* will yield more biologically and clinically relevant data. Cells that are grown as tissue like structure will be in close contact with each other simulating the generation of a specific microenvironment and the close cell to cell contact will provide a more natural cellular profile affecting all intracellular processes [117, 119, 120].

Table 1.6.1 Examples of commercially available technologies for generating *in vitro* 3D structures.

Company	Trade name	Type and material
3DBiomatrix	Perfecta3D plates	Hanging drops
	Perfecta3D scaffolds	Hydrogel
BD	Matrigel	Laminin, collagen
Glycosan Biosystems	Extracel	Hyaluronic acid and collagen
GlobalCellSolutions/ Hamilton	GEM	Magnetic alginate microcarrier
Trevigen	Cultrex 3D Matrix	BME, laminin, collagen
Sigma	HydroMatrix	Synthetic peptide hydrogel
	MaxGel	Human ECM
Qgel	MT 3D Matrix	PEG hydrogel
Synthecon Inc.	BIOFELT	PGA, PLLA, PLGA, custom
	Biomerix 3D Scaffold	Polycarbonate polyurethane-urea
ZellWerk	AlgiMatrix	Alginate
	Sponceram	Ceramic
Amsbio	Cultrex	Basement Membrane Extracts
	Alvetex	Polystyrene
3DM Inc.	PuraMatrix	Peptide
Corning	UltraWeb	Polyamide
MicroTissues Inc.	3D Petri Dish	Agarose
Biocroi	Happy Cell	Liquid scaffold
3DBiotek	3D Insert PCL	Polycaprolactone
	β -TCP Disc	β -Tricalcium phosphate

There are many approaches to generating 3D tumoral structures *in vitro* (Table 1.6.1) from solid scaffolds to which cells are added [116, 117], novel plates that allow cells to grow suspended in medium, where cells naturally aggregate into spheroids (tumour like structures) [120] to new mediums that act as a scaffold yet remain in a liquid state. These spheroids can be generated from a single cell type (either from immortalised commercially available cell lines or isolated from patient biopsies [121]) (Fig. 1.6.1 A) or they can be generated from two or more cell types (Fig. 1.6.1 B), in this case cells can naturally or be chemically induced to generate layers (Fig. 1.6.1 Bi) of defined structures (such as vessels) that can be seen *in vivo* (Fig. 1.6.1 Bii).

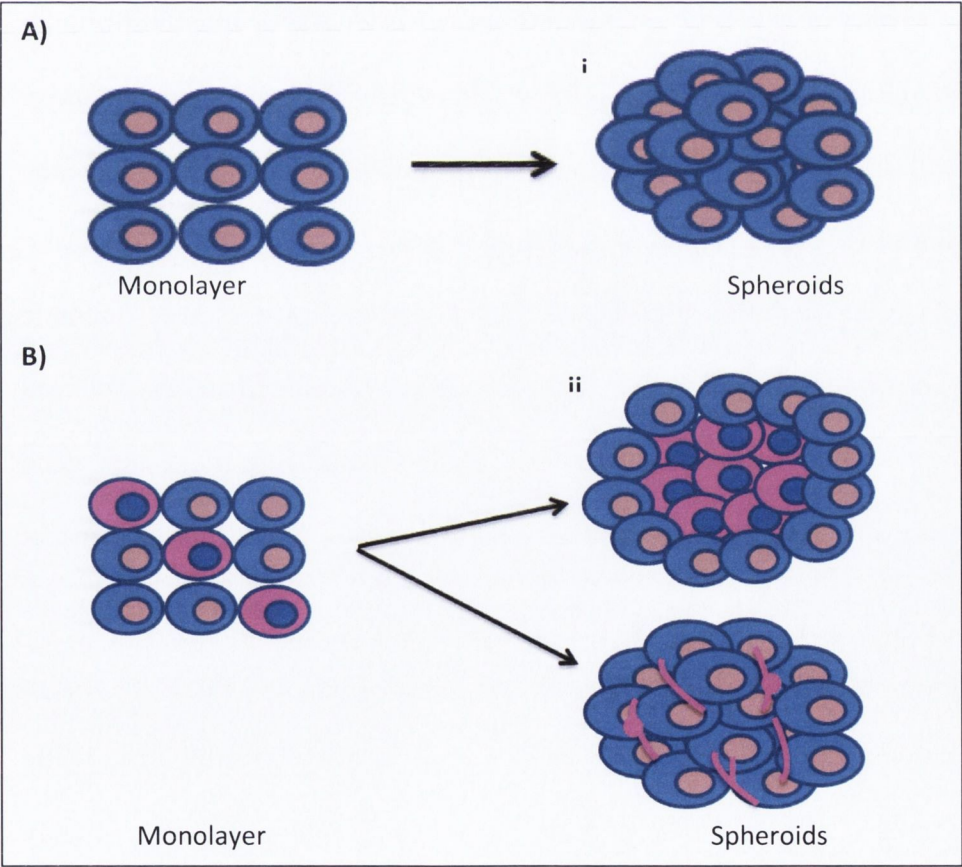


Figure 1.6.1 Schematic representation of different types of spheroid generation. A) A single population of cells will form a solid mass of cells. B) A mixed population of cells can form two types of tumours where one of the cell lines is internalized (i) or one of the cell lines turns into external structures surrounding the tumour-like mass (ii).

There are three characteristics which are central for the successful engineering of 3D structures:

1. Co-localization of cells, followed by formation cell to cell interactions, interchange of growth factors and other biologically relevant molecules [122].
2. Production of an external cell matrix that provides not only a physical scaffold but also regulates cellular functions [122].
3. Production of interstitial fluid containing, in its composition, the necessary nutrients and biological effectors for tissue differentiation, maturation, proliferation and regulation [122].

In drug development the pre-animal testing in these tumour like multicellular structures plays a pivotal role as it is known that most cells growing in monolayer form will be more susceptible to toxicants; both to potential therapeutic compounds [118, 123] and vehicles of drug delivery.

It is known that cells in a three dimensional structure more closely resemble natural solid tumours. Cell to cell adhesions, the extracellular matrices produced in this type of architecture and hypoxic regions within the mass alter gene expression inducing different metabolic pathways as well as altering nutrient availability and waste removal from any region of the spheroids [118]. This leads to a stratified architecture where the outer cells of the spheroid are in proliferation followed by a layer of cells that lie in a more resting state while regions in the centre tend to be necrotic due to hypoxia and waste accumulation [118]. This heterogeneity closely resembles that found in solid tumours [124] and gives rise to many of the issues associated with cancer. For example drug

resistance, issues completely eliminating tumours and production of metastasis [124].

In order for PDT to have the required effect, it requires a minimum amount of oxygen to be present in tissues [125, 126]. In addition PDT treatments have, as a consequence, the local depletion of oxygen [126]; this added constraint to this therapeutic modality reflected on the fact that the effect of PDT in dysplastic tissue is abolished when oxygen is absent [126] presenting a major challenge when dealing with anoxic regions of tissue. Nonetheless PDT requires only a small amount of oxygen to be present in order to exert its effect and in areas of reduced oxygen the fluence rate of the light delivered must be adjusted in order to maximise tumour destruction [127].

It has been implied that testing potential drugs in spheroids rather than monolayers would have a more predictive value of toxicity in patients than tests in monolayers [128]; however, the development of consistent 3D model for implementation in high throughput toxicity testing is yet to become reality [129-131].

Imaging these structures poses many issues as most microscopes have limited ability to penetrate three dimensional complex structures. The most common method of evaluation structures of this type would be fluorescent imaging either in a fluorescent widefield or confocal microscope.

1.7 Luminescence bioprobes

Central to the technique of fluorescent imaging (and consequently HCS technologies) lie a large range of molecules that are able to localize and accumulate in specific locations whether intracellularly or in a specific tissue and are fluorescent in nature [132]. These molecules are commonly used in many

biological fields from cancer research to more complex medical investigations [132, 133].

Today fluorescence imaging is routinely used in laboratories and clinics around the world since it is such a robust and versatile technique. It can be used in fixed tissues/cells as well as in live tissues/cells, and in living organisms because it is minimally invasive and its toxicity is minimal [133-135].

By definition a fluorophore is a molecule that absorbs light at a particular wavelength temporarily exciting the molecule; light is then emitted a longer wavelength when the molecule returns to its ground state, this is a phenomenon known as Stoke's shift [136, 137]. This emitted light can then be detected by a microscope, spectrophotometer etc. in order to quantify a certain parameter (localization, viability, among many others). There is a wide range of commercially available fluorophores ranging from small synthetic molecules [138] to naturally occurring compounds (such as porphyrins) to more complex constructs such as antibodies labelled with fluorescent molecules (e.g. anti-Golgi antibodies labelled with FITC) and cells or organisms labelled with GFP or other genetically engineered fluorescent proteins [139] which are genetically inserted into cells and expressed *in vivo* [139] (Fig.1.7.1).

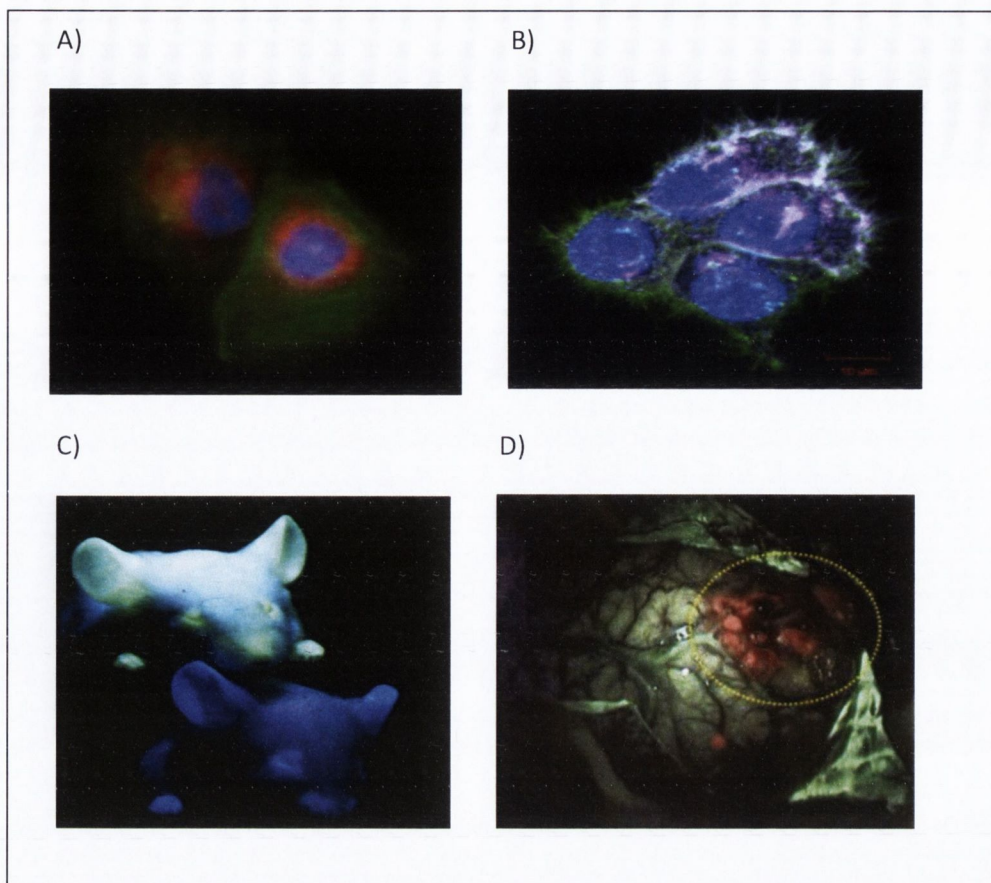


Figure 1.7.1 Cell and organisms expressing fluorescence. A) Cells labelled with Hoescht (blue nucleus), green phalloidin (F-actin) and red Temoporfin (cytoplasm) [140]. B) Immunocytochemistry for transferrin receptor (red). F-actin was stained with FITC-labelled phalloidin (green) and cell nuclei were counterstained with Hoechst 33342 (blue) [141]. C) Mice expressing yellow fluorescent protein (YFP) (top) and cyan fluorescent protein (CFP) (bottom) [142]. D) Double therapeutic and diagnostic technique used to treat and remove malignant gliomas, patient was injected with 5-ALA prior to surgery; during surgery the tumour is illuminated allowing identification of the tumour (in red), tumour is then removed by ablation with a laser that also activates the PDT effect destroying any remaining tumoural cells [143].

Fluorophores in biological research have to fulfil two broad requirements, they must consistently absorb light in a particular wavelength of the spectrum as well as emit at a separate wavelength and they must target a particular location in the cell or tissue [144]. They are so ubiquitous in science that it is an ever expanding field with many commercially available dyes, ranging from small molecules that bind to DNA (Table 1.7.1) to more complex constructs such as phalloidin labelled with various fluorescent

molecules (such as Alexa Fluor 488, 535, etc.), antibody fluorophore complexes further target the fluorescent to specific locations in cells and tissues; There are also many others in development [145, 146].

Table 1.7.1 Examples of commonly used DNA fluorescent probes [144].

Dye Name	Excitation (nm)	Emission (nm)	Live / Fixed applications
Hoechst 33342	350	461	Live and fixed cell based assays
DAPI	358	461	Fixed cell based assays
Propidium iodide	530	625	Fixed, permeabilized, dead cell based assays
Draq 5	647	670	Fixed, permeabilized and live cell based assays
Draq 7	633	694	Fixed, permeabilized, dead cell based assays
TOTO3	640	660	Fixed, permeabilized, dead cell based assays

In a laboratory setting fluorescent probes are commonly used to detect many parameters including cell viability (propidium iodide and Draq7 are commonly used in both microscopy and flow cytometry platforms) [147-151]; cell morphology (fluorescent labelled phalloidin and paclitaxel constructs are used to stain F-actin and tubulin respectively [152-155]). DNA alterations (fluorescent microsatellite analysis can be used to easily quantify genome-wide

DNA changes) [156, 157]. Fluorescent *in situ* hybridization (FISH) uses highly specific complementary DNA or RNA sequences tagged directly with fluorophores, antibodies or biotin to detect DNA abnormalities [158, 159]. These are just a few examples of fluorescence being applied in a biological laboratory. Many of these experimental protocols have been also adopted by clinical laboratories, for example, a FISH approach is now used to diagnose unexplained male infertility [160] and for the diagnosis of pre natal aneuploidies [161]. Intravenous fluorescein angiography is routinely used in a clinical setting; this diagnostic tool uses sodium fluorescein to diagnose any problems related to the vasculature of the retina [162].

1.8 Aims and objectives

To setup and validate a semi automatous HCS method for the assessment of toxicity of photodynamic porphyrins as possible PDT agents.

With this newly established assay we aim to assess a number of different porphyrin libraries to test the consistency of the assay and from this primary screen retrieve a number of hits, going further with these using other toxicity tests.

Generate 3D spheroids using Happy Cell® and test a panel of porphyrins comparing the results to 2D experiments.

Finally a panel of novel small fluorescent molecules will be evaluated for their suitability for fluorescent labels in HCS and confocal technologies as well as determining their, if any, toxic effects on cells.

CHAPTER TWO:

Materials and Methods

2.1 Cell lines

Four different types of cell lines were studied in order to represent a wide scope of oesophageal malignancies:

- OE21 – Human oesophageal squamous carcinoma cell line also known as JROECL21 [163, 164].
- OE33 – Human oesophageal adenocarcinoma cell line of the lower oesophagus also known as JROECL33 [164, 165].
- SKGT-4 – Human oesophageal adenocarcinoma of Barret's epithelium of the distal oesophagus exhibiting minimal invasive potential [166].

A fourth cell normal oesophageal cell line was used in the studies for potential bioprobes:

- HET-1A – immortalized normal oesophageal epithelium [167].

All cell lines were purchased from the European Collection of Cell Cultures (ECACC).

2.2 Cell culture

The immortalized Human normal oesophageal epithelial cell line Het-1A [167] was routinely cultured using an antibiotic free BEGM kit (Lonza, UK) in cell culture flasks (Nunc, Denmark) and split 1:4 until 70-80 % confluency was reached. Cell recovery was achieved using 0.25% Trypsin with 0.1% EDTA in hanks balanced salt solution (Hyclone, USA) for two minutes at 37 °C and 5% CO₂ in humidified atmosphere. The trypsin was inactivated using a 1:1 0.1% soybean trypsin inhibitor (Sigma, Germany). The culture medium was changed every three days until confluency was obtained.

All other cell lines were cultured in RPMI 1640 (Hyclone, USA), supplemented with 10 % inactivated fetal bovine serum (Hyclone, USA) and 1 % penicillin/streptomycin (Hyclone, USA). Cultures were routinely grown in cell culture flasks (Nunc, Denmark) and split 1:8 until 70-80 % confluency was reached. Cell recovery was achieved using 0.25% trypsin with 0.1% EDTA in hanks buffered salt solution (Hyclone, USA) for three minutes at 37 °C and 5% CO₂ in humidified atmosphere and the culture medium was changed every three to four days until confluency was obtained.

2.3 High content screening assay development and screen of porphyrin libraries

2.3.1 Treatments

For photodynamic treatment and uptake studies 3×10^3 OE21 cells per well were plated in 96-well plates (Nunc, Denmark) and treated with either 3 μ M, 2 μ M, 1 μ M, or 0.5 μ M of Temoporfin solution in the dark for up to 24 hours at 37 °C and 5% CO₂. Control cells were treated with vehicle alone. The Temoporfin and glycoporphyrin solutions were prepared by dissolving 5,10,15,20-tetrakis(3-hydroxyphenyl)chlorin, synthesized following standard procedures [168], and (5,10-di- β -glucose2,-porphyrinato)zinc(II) in ethanol:propylene glycol = 3:2 (v/v) (Acros organics, USA). All other compounds tested were dissolved in 100% dimethyl sulfoxide (DMSO) (Acros organics, USA), at 2 μ M and the identified hits were then used to treat cells in the concentrations previously discussed. The equivalent amounts of DMSO and of the ethanol and propylene glycol mixture were also assessed for toxicity (up to a concentration of 0.48% (v/v)). A final screen of 22 compounds dissolved in DMSO was performed in OE21, SKGT-4 and OE33 cell lines using a single concentration of 2 μ M. In this

experiment OE33 and SKGT-4 were plated at a concentration of 6×10^3 cells per well and OE21 were plated at a concentration of 3×10^3 cells per well.

The identified hits were then prepared fresh in DMSO at a concentration of 10mM and then tested at 24 μ M, 12 μ M, 6 μ M, 3 μ M, 2 μ M, 1 μ M and 0.5 μ M.

2.3.2 Illumination protocol

Following the removal of the treatment medium (medium with potential drug at the desired concentrations) and addition of fresh, pre-heated, fully supplemented RPMI the incubated plates were separated into two groups. Group 1 was illuminated for two minutes using an illumination box, consisting of Luxeon High Power LEDs (LXHL-BW03) as light sources emitting white light with a total fluence rate of 1.7 mW/cm⁻² [169]. Group 2 was kept in the dark under the same conditions as group 1. The cells were allowed to recover for four hours in the dark at 37 °C and 5% CO₂ in a humidified atmosphere.

2.3.3 Cell proliferation assay

Two plates (one illuminated and one non-illuminated) were used for a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [170] (Promega, USA). Immediately, following illumination 15 μ L of MTT dye was added to each well and kept for four hours at 37 °C and 5% CO₂ in a humidified atmosphere in the dark at which point 100 μ L of the provided stop solution was added to each well. The formazan crystals were allowed to dissolve overnight at room temperature and absorbance was measured at 540 nm with a Wallac Victor2 plate reader (Perkin Elmer, Singapore).

Final dose-response experiments were performed using the same plate setup but analysis was done with a MTS kit (Promega, USA). These plates were allowed

to recover for 20 h at which point 20µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) dye (mixed with phenazine methosulfate (PMS) immediately prior to addition to the wells) was added to each well. Plates were incubated for a further 4 h then immediately read at 490 nm with a Biotek EL800 (BioTek, USA).

All development experiments were performed according to the Fig. 2.3.1

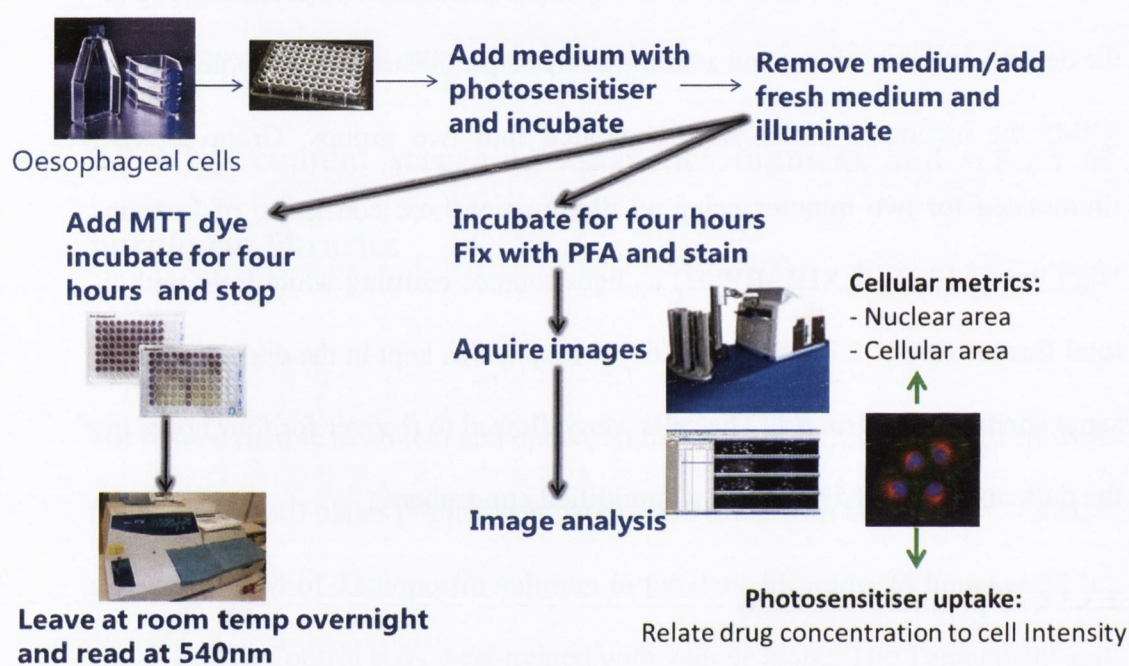


Figure 2.3.1 Flow scheme of experimental protocol. Representing both MTT assay methodology as well as HCS protocol (method development).

2.3.4 High content imaging and analysis

Following a four hour post illumination recovery, two plates (one illuminated and one non-illuminated) were fixed with 4 % paraformaldehyde solution (PFA) (Acros Organics, USA), pre-heated to 37° C for 15 minutes and further stained with FITC-labeled phalloidin (Dyomics, Germany), an F-actin stain, and Hoechst (Invitrogen, USA), a nuclear stain. Images were collected using the

InCell 1000 high content system (GE Healthcare, USA). A total of ten fields per well were imaged under 10× magnification using three separate filters to capture the nucleus (blue, excitation 345 nm, emission 435nm), F-actin (green, excitation 475 nm, emission, 535 nm) and Temoporfin (red, excitation 560 nm, emission 700 nm). In order to obtain information about cell number a further seven fields (covering the vast majority of the well) were obtained at 4× magnification using a single blue filter, collecting nuclear fluorescence alone.

Image analysis was performed using the InCell 1000 image analyser using three different algorithms according to the parameters needed. In order to determine cell and nuclear area a morphology algorithm was used, while for photosensitiser parameters a dual area object analysis algorithm was used and a multi target analysis algorithm was used in a separate set of images (4× objective) in order to determine cell number (Table 2.3.1).

Table 2.3.1 Description of parameters used in the image analysis performed using the InCell investigator software.

Parameter	Description
Nuclear area	Area of identified cell nucleus
1/form factor	Mean cell roundness index. Values range from 1 to infinity where 1 is a perfect circle (elongation of cell)
Cell area	Area of identified cell body
Cell number	Number of identified cell nuclei
PS integrated intensity	Average fluorescence intensity of pixels within the cytoplasmic region of the red PS multiplied by the area of identified red fluorescence
PS area	Area of identified intracellular red fluorescence

The image analysis software detects cells nuclear area/cell number by nuclear dye uptake with quantifications of cell morphologies and PS parameters determined by F-actin stain and Temoporfin. Stain parameters such as morphology and intensity of fluorescence were logged numerically for individual cells in every field as well as averages for each field and well. All development experiments were performed according to Fig. 2.3.1

2.4 Assay application to various porphyrin libraries

Different libraries were investigated. All compounds were dissolved in DMSO to a 10 mM concentration. All concentrations tested were then prepared in fresh complete RPMI in the various necessary concentrations and added to the cells; all tests were performed according to one or more of the previously described protocols.

2.5 3D experiments

2.5.1 Assessment of spheroid formation

OE21, SKGT-4 and OE33 cell lines were used to assess the potential to form spheroids in RPMI Happy Cell (Biocroi, Ireland). Different concentrations of cells (1.5×10^4 , 3×10^4 , 7.5×10^4 and 15×10^4 cells per well) were seeded into 96 well low adherence plates (Sarstedt, Germany) and left for up to 7 days. Assessment of spheroid formation was done by visual inspection, images were collected with an EVOS® FL digital fluorescence microscope (LifeSciences, USA) with a 4× objective.

2.5.2 Viability of spheroids following PDT

OE33 cells were plated into 96 well low adherence plates (Sarstedt, Germany) at a concentration of 7.5×10^4 cells per well, in Happy Cell in parallel. The spheroids were allowed to form and mature for 72 hours at 37 °C with 5% CO₂. All cultures were treated with Temoporfin, a negative control and test porphyrins. These compounds were made in fresh vehicle, as previously described, to a concentration of 5mM. These were added to the spheroid or monolayer cultures in fresh Happy Cell or RPMI to yield a final concentration of 12μM; vehicle controls were tested in parallel.

The various treatments were allowed to exert their effects for 24 hours. At 22 hours the Happy Cell was inactivated using 10μL of Inactivation Solution (Biocroi, Ireland) per 100 μL of Happy Cell and incubated for 2 h at 37 °C with 5% CO₂.

At this point most of the solution was removed from the wells; 50μL of solution was left in each well insuring the majority of spheroids were left in the well for further testing. 100μL of fresh RPMI or Happy Cell were added to each respective well. Then, one plate was illuminated (according to the illumination protocol previously described) and the other was kept in the dark.

The spheroids were allowed to recover for a total of 24 hours. At hour 20, 30μL of MTS were added to each well, according to manufactures instructions. The MTS developed for 4 hours and plates were read at 490 nm with a Biotek EL800 plate reader (BioTek, USA).

2.5.3 Fixation and further staining

Parallel treated plates were fixed in 2% PFA (Acros Organics, USA) for 24 hours, PFA was then removed and cells were washed with PBS and further

stained with Hoechst (Invitrogen, USA). Spheroids were transferred to glass bottom black walled plates (GE, USA) and evaluated in an LSM 700 Confocal Laser Scanning Microscope (Carl Zeiss, UK) using a red laser (for porphyrin uptake) and a blue laser (for Hoescht stain) at 63× oil immersion objective.

2.6 Assessment of small molecules as potential fluorophores

A 1 mM stock solution was prepared in DMSO for each compound. Dilutions were carried out directly before starting the analyses. For cell stain imaging a stock solution prepared in DMSO was diluted with phosphate buffer saline to different concentrations. Buffer solutions prepared at the desired concentrations were added directly to the cell culture. After adding compounds, cells were incubated at 37 °C in 5 % CO₂ for 20 minutes to 24 hours. All tests were performed in 3×10³ cells per well of OE21 and 6×10³ cells per well HET-1A cells that were allowed to attach overnight prior to any tests.

2.6.1 Live cell testing

The cells were treated with fresh pre-heated medium containing various concentrations of compounds and empty carrier was used as control. The cells were allowed to take up the compounds for up 24 h at which point the medium was removed and replaced with fresh pre-heated medium and fixed with 4% paraformaldehyde. These cells were then analysed in the InCell 1000.

2.6.2 Fixed cell testing

Cells were fixed with 4% paraformaldehyde, washed twice with PBS and incubated with various concentrations, in PBS, of the compounds for different

times. The cells were then further stained with Draq5 (Biostatus, United Kingdom). These cells were then analysed in the InCell 1000 for fluorescence.

2.6.3 Confocal imaging

Cells were seeded into glass bottom sterile plates (GE Healthcare, USA) and allowed to attach overnight. An ER tracker red probe (Invitrogen, USA) was added to the cultures according to manufactures instructions and these were either fixed with 4% paraformaldehyde (Acros Organics, USA) and analysed or incubated live with the compounds then fixed and analysed in LSM 700 Confocal Laser Scanning Microscope (Carl Zeiss, UK).

2.7 Confocal image collection

Cells were seeded into glass bottom sterile plates (GE Healthcare, USA) and allowed to attach overnight. All confocal images were collected using a LSM 700 confocal laser scanning microscope (Carl Zeiss, UK). Under a 63× oil immersion objective.

2.8 Statistical analysis

Each experiment was repeated a minimum of three times and results were normalized to untreated controls. Averages and standard error of the mean (SEM) of each well were plotted using Graphpad Prism version 5.0 (GraphPad Software, USA). Differences in the cell proliferation data were evaluated by ANOVA analysis with Bonferroni comparison. Correlation between InCell and MTT data was evaluated by Pearson correlation test with a 99 % confidence.

Regressions (linear and non-linear) were fitted to the photosensitiser data (intensity and area). All statistical analyses were performed using Graphpad Prism version 5.0. Analysis of Variance (ANOVA) statistical analysis and T-tests were performed when necessary with a 95% confidence interval using Graphpad Prism 5 software (GraphPad Software Inc., USA).

CHAPTER THREE:

Results and Discussion

3.1 In Cell 1000 experiments

The Incell 1000 system, in its core, has integrated a powerful fluorescent microscope in order to collect and store images or experimental models, being that of cells, tissues or complete organisms. Any naturally fluorescent molecule could be assessed using this system; porphyrins, in particular with their fluorescence (Fig. 3.1.1) should be easily imaged using this platform.

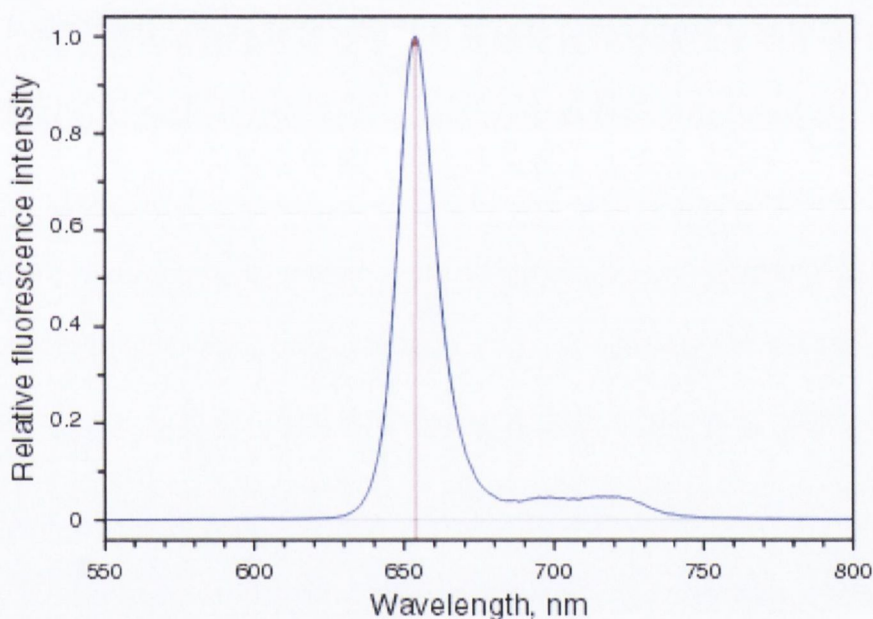


Figure 3.3.1 Graphic representation of the emission profile of Temoporfin in polyethelene glycol and ethanol at 2.5×10^{-5} M [93].

Yet, and as a first step, it was necessary to verify if photosensitisers could be used in the In Cell 1000 as a fluorescent marker. From Fig. 3.1.2 it is clear that the fluorescent signal produced by Temoporfin is, in fact detected in the In Cell 1000. It can also be seen that if the concentration of Temoporfin is too high the system is saturated e (Figure 3.1.2).

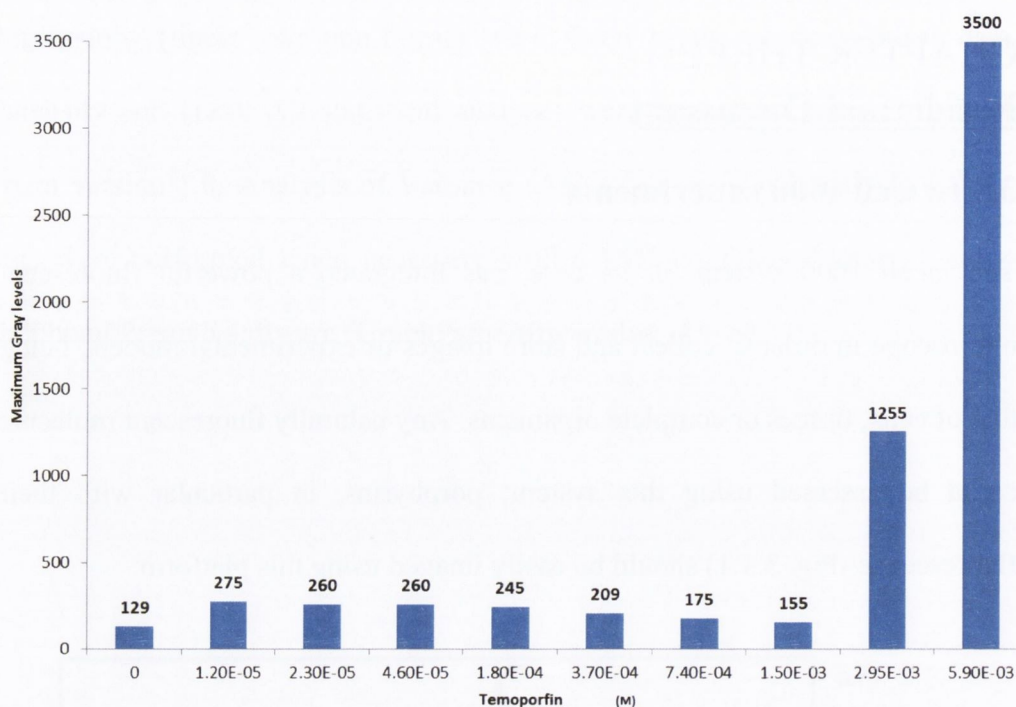


Figure 3.1.2 Graphic representation of various concentrations of Temoporfin in the In Cell 1000.

As temoporfin fluorescence could be measured using the InCell 1000 system, development of a one-step fluorescent assay for the assessment of phototoxicity and uptake parameters of porphyrins and possible photosensitisers could continue.

3.2 High content screening assay development

Automated imaging has been available for the last 20 years and it has significantly streamlined the process of *in vitro* drug screening in addition to decreasing the time needed both for high quality image collection and analysis [171, 172]. Naturally fluorescent drugs have, in the past, been evaluated by fluorescent microscopy [173-176]. However, a phenotypic screen of toxicity in cells using naturally fluorescent compounds has seldom been attempted due to its inherent restrictions. Nonetheless, here the results obtained for a novel high content assay developed using fluorescent drugs in OE21 cells, a human

oesophageal squamous cell carcinoma cell line derived from a human carcinoma and SKGT-4 cells, a human adenocarcinoma of the oesophagus derived from a human adenocarcinoma caused by Barrett's oesophagus are presented

3.2.1 Cell proliferation

MTT measures the proliferation of cultures through an estimation of mitochondrial activity; this is achieved by the reduction of MTT into formazan. The reduction is performed by the action of mitochondrial dehydrogenases in metabolically active cells; the amount of formazan produced is known to be directly proportional to the mitochondrial enzymatic activity and, therefore, to the number of proliferating cells in culture [177-180]. This assay has been extensively used both to determine cell proliferation, viability and to determine toxicity of potential therapeutic agents [181]. First, the effect of Temoporfin on OE21 and SKGT-4 cells was tested with the MTT test in order to have a classical toxicity result with a widely used technique. As shown in Fig. 3.2.1 there was a decrease in cell viability upon plate illumination with increasing Temoporfin concentrations both for OE21 and SKGT-4 cells. No dark or vehicle toxicity was observed. In the case of OE21 cells the two way ANOVA analysis showed a statistically significant difference between illuminated and non-illuminated data where Temoporfin treated cells showed a statistically significant difference from concentration 2 μ M up. The same was observed for SKGT-4 cells. The same statistical analysis showed that, for both cell lines, vehicle treated cultures showed no such difference in the presence or absence of light. These results are in accordance to the literature where Temoporfin alone or vehicle were shown

to have no effect but Temoporfin treated cells in the presence of light showed significant toxicity in a dose dependent manner [168, 182, 183].

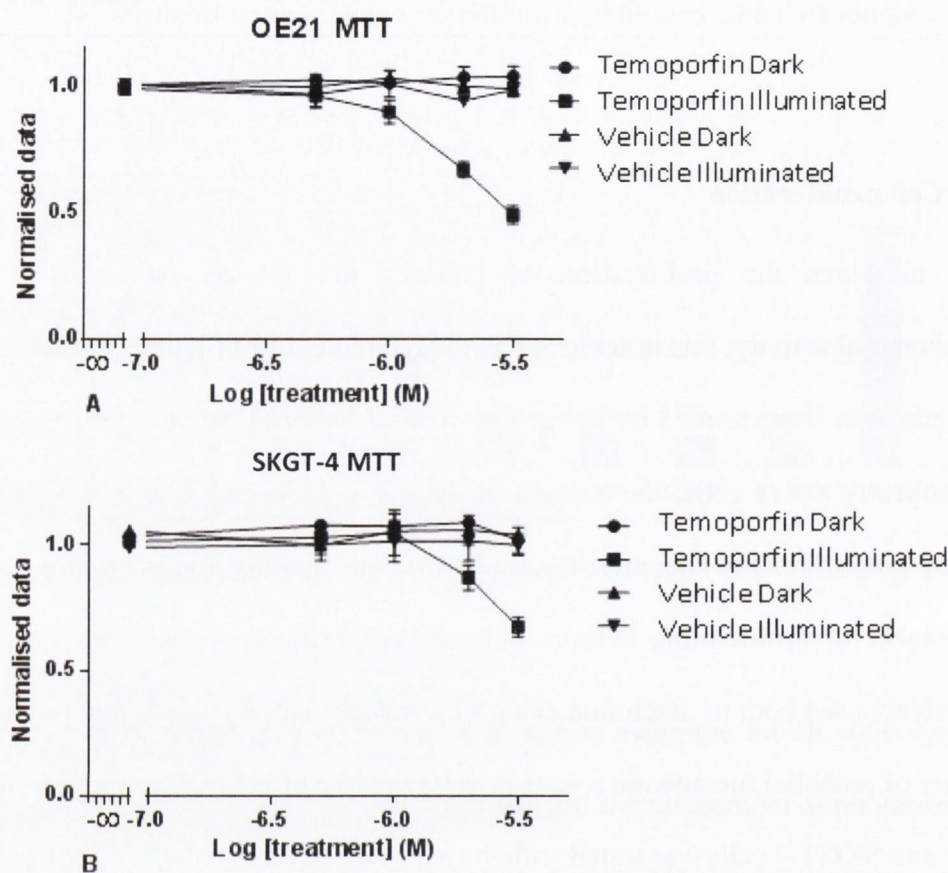


Figure 3.2.1.1 OE21 (A) and SKGT-4 cells (B) treated with increasing concentrations of Temoporfin and with equivalent concentrations of vehicle alone (A and B) (illuminated and non-illuminated) were assessed for toxicity using a traditional cell proliferation assay (MTT). Data are representative of four (OE21) and three (SKGT-4) independent experiments and values are expressed in mean $\pm$ SEM.

3.2.2 High content analysis

Next we used an HCA platform to analyse the morphological changes that occur upon PDT treatment of OE21 and SKGT-4 cells. Cell death results in many changes of cellular morphological characteristics and these may be used for HCA analysis. Fig. 3.2.2.1 shows some examples of images collected using the HCA platform of OE21 cells. Clearly, illumination in the presence of the photosensitiser results in dramatic changes to the cells compared to the dark

control. The severity of these physical cell deformations increased with the Temoporfin concentration. Similar changes occurred in SKGT-4 cells (not shown).

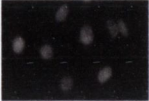
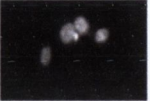
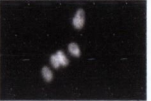
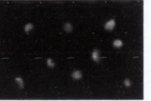
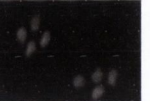
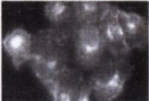
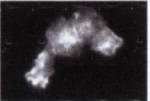
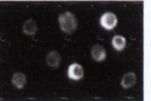
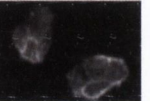
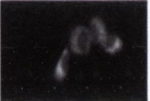
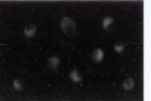
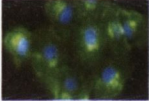
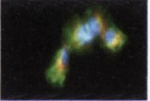
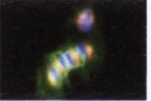
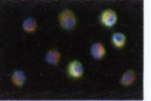
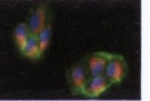
	Untreated Dark	3µM Temoporfin	0.5µM Temoporfin	3µM Temoporfin Illuminated	0.5µM Temoporfin Illuminated
Nucleus (Blue)					
F-Actin (Green)					
Temoporfin (red)					
Superimposed Image					

Figure 3.2.2.1 Examples of images collected with the InCell imaging system for the different treatments used with OE21 cells treated with 3µM Temoporfin for 24 hours before illumination and fixed at 4 four hours post illumination in 4% paraformaldehyde. Cells were labelled with Hoechst – nucleus (blue at 345 nm excitation, 435 nm emissions), phalloidin –F-actin (green at 475 nm excitation and 535 nm emission), *m*THPC (red) was acquired at 560 nm excitation and 700 nm emission. Images were acquired by an InCell analyser automated microscope using a 10× objective (image size 0.897 mm×0.671 mm).[184]

The earliest phenotypic signs of cell toxicity are reflected by changes in nuclear size, cell fragmentation, and significant changes in cell area and a cell shape [185]. Other phenotypic changes include cell membrane breakdown, leakage of cellular contents and increased numbers of cytosolic, lysosomal vesicles. Clearly an automated approach to the quantification of these features can be useful during a screen of large numbers of compounds by taking advantage of these

marked changes in cell morphology. Typically, changes in nuclear morphology are amongst the first effects seen during cell death [80].

Results showed that the nuclear area in OE21 cells in fact reflects the phototoxicity of Temoporfin (Fig. 3.2.2.2). Not only does nuclear area reflect toxicity in a dose-dependent fashion (Fig. 3.2.2.2 A), where it is clear that nuclear area was reduced with increasing concentrations of Temoporfin, but it followed this trend in the same fashion as seen in the cell proliferation assay (Fig. 3.2.2.2 B). The two way ANOVA analysis revealed that when comparing dark nuclear area and illuminated nuclear area there was a significant difference in the data up to, but not including, the 1 μ M dose. On the other hand, comparison of MTT in the dark with illuminated MTT data showed a significant difference in the data up to, but not including, 1 μ M of Temoporfin.

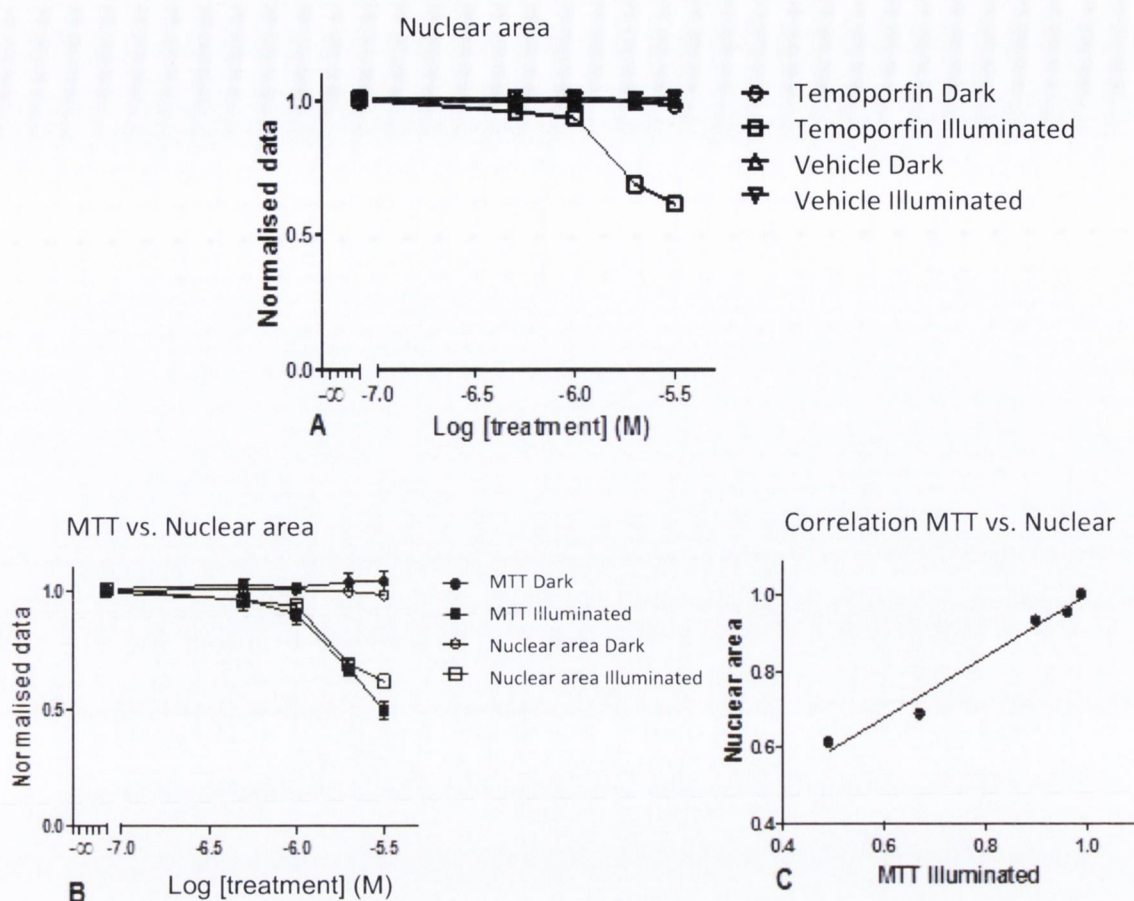


Figure 3.2.2.2 Graphical representation of the nuclear area data generated using the InCell image analysis in OE21 cells. Representation of the nuclear area with increasing concentrations of Temoporfin and with equivalent concentrations of vehicle in illuminated and dark cells (A). Data generated by MTT were compared to InCell data (B). The data generated for illuminated cells were compared in order to establish the degree of correlation (C). Data are representative of four independent experiments and values are expressed in mean $\pm$ SEM.

A comparison of MTT in the dark with nuclear area in the dark, showed no statistically significant difference in the data at any of the concentrations tested. However, a comparison of their illuminated counterparts indicated a significant difference in the data at 3 μ M. This difference was not observed at any other concentration. In order to evaluate if the trends observed in the nuclear area data and MTT data were related, a Pearson correlation test was performed. This confirmed a very high correlation between the MTT results and the nuclear area

with $P=0.0009$ with a 99 % confidence interval between 0.59 and 0.99 (Fig. 3.2.2.2 C). There is also a clear decrease in data variability as indicated by a lack of SEM bars in Fig. 3.2.3A.

This parameter, however, failed to reveal any relevant toxicity information in SKGT-4 cells as it mostly remained unchanged (data not show). Nonetheless, the large number of output parameters from the image analysis indicated that the parameter 1/form factor can yield significant information about the toxicity of Temoporfin in SKGT-4 cells. This cell line differs much in appearance to OE21 cells. They have a more stretched appearance and the phenotype changes significantly upon treatment with Temoporfin; the cells became much more round in shape than their untreated and non-illuminated counterparts. The results show that the 1/form factor in SKGT-4 cells in fact reflects the phototoxicity of Temoporfin in a similar fashion as does the nuclear area for OE21 (Fig. 3.2.2.3 A). Likewise, no toxicity was observed upon treatment with vehicle alone (Fig. 3.2.2.3 A). The parameter reflects toxicity in a dose-dependent fashion and follows the same trend seen in the cell proliferation assay (Fig. 3.2.2.3 B). Further ANOVA analysis showed a significant difference in the comparison of dark 1/form factor and illuminated 1/form factor there in the data up to, but not including, the 1 μ M dose. A similar result was obtained for the MTT data. When comparing MTT in the dark with 1/form factor in the dark, there was no statistically significant difference in the data at all concentrations tested. However, a comparison of their illuminated counterparts showed a significant difference in the data at 1 μ M. At all other concentrations this difference was not observed. Once again, the 1/form factor data and MTT data were tested for correlation by using a Pearson correlation test. Here the correlation between the MTT results and 1/form factor was high with $P=0.00271$ with a 99 % confidence

interval -0.45 to 0.99 (Fig. 3.2.4C). Similarly, a clear decrease in data variability was observed, this is clearly represented by the SEM bars in Fig. 3.2.2.3 A.

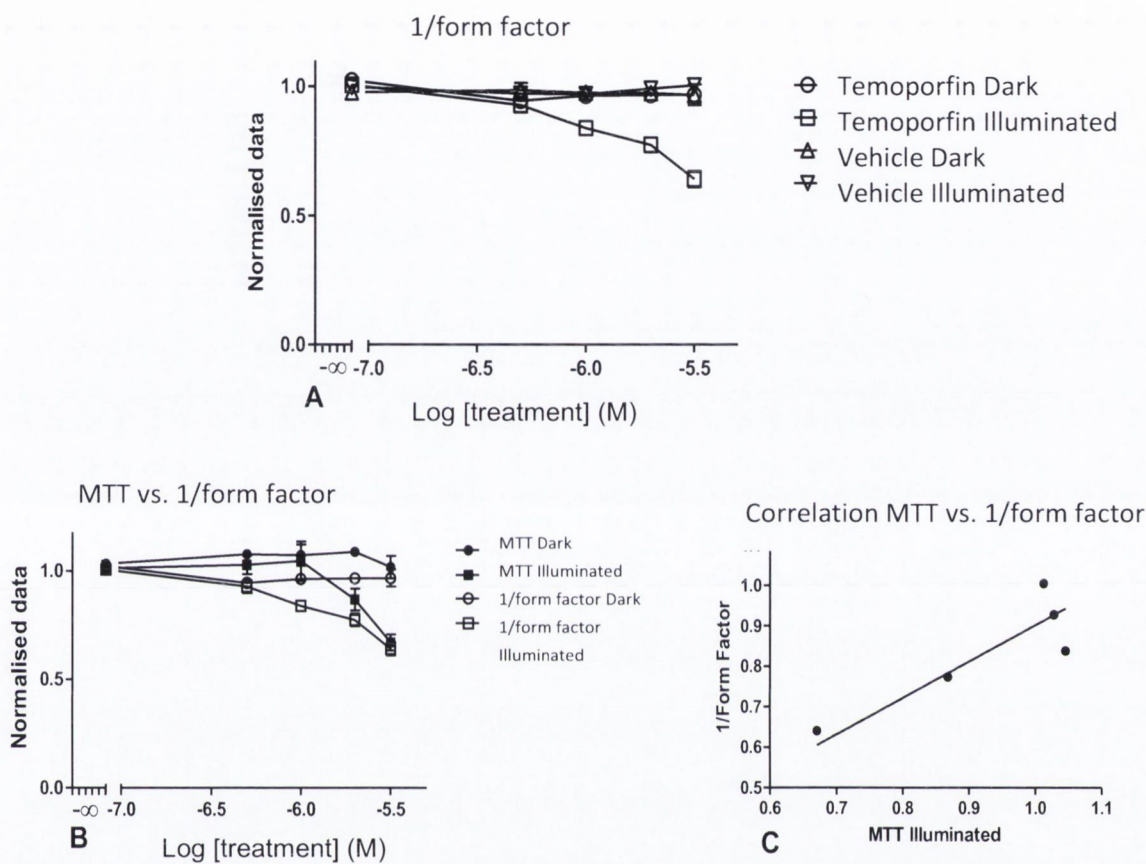


Figure 3.2.2.3 Graphical representation of the 1/form factor data generated using the InCell image analysis (A and B) in SKGT-4 cells. Representation of the 1/form factor with increasing concentrations of Temoporfin and with equivalent concentrations of vehicle (A) in illuminated and dark cells (A). Data generated by MTT were compared to InCell data (B). The data generated for illuminated cells were compared in order to establish the degree of correlation (C). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

Another morphological parameter to change upon PDT treatment is the cellular area (Fig. 3.2.2.4 and 3.2.2.5). Note, that the images in Fig. 3.2.2.2 indicated that the cell area was severely affected by the action of Temoporfin and light. For OE21 cultures the graphical representation of the data showed a significant reduction of cell area with increased concentrations of Temoporfin, yet, showed

no difference in cells treated with vehicle alone and those kept in the dark (Fig. 3.2.2.4 A). This particular parameter showed the same trend as the classical MTT assay (Fig. 3.2.2.4 B) and seems to be slightly more sensitive at detecting toxicity. Again, there is a very high correlation between the data generated by detecting cell area and the MTT assay (Fig. 3.2.2.4) with $P = 0.0014$ with a 99% confidence interval in the 0.49 to 0.99 range (Fig. 3.2.2.4 C). The ANOVA test showed no statistically significant difference between illuminated to dark cell area at all the Temoporfin concentrations tested. Conversely, there were no statistically significant differences between MTT and cell area regarding cultures kept in the dark, and the same result was obtained when testing illuminated cultures.

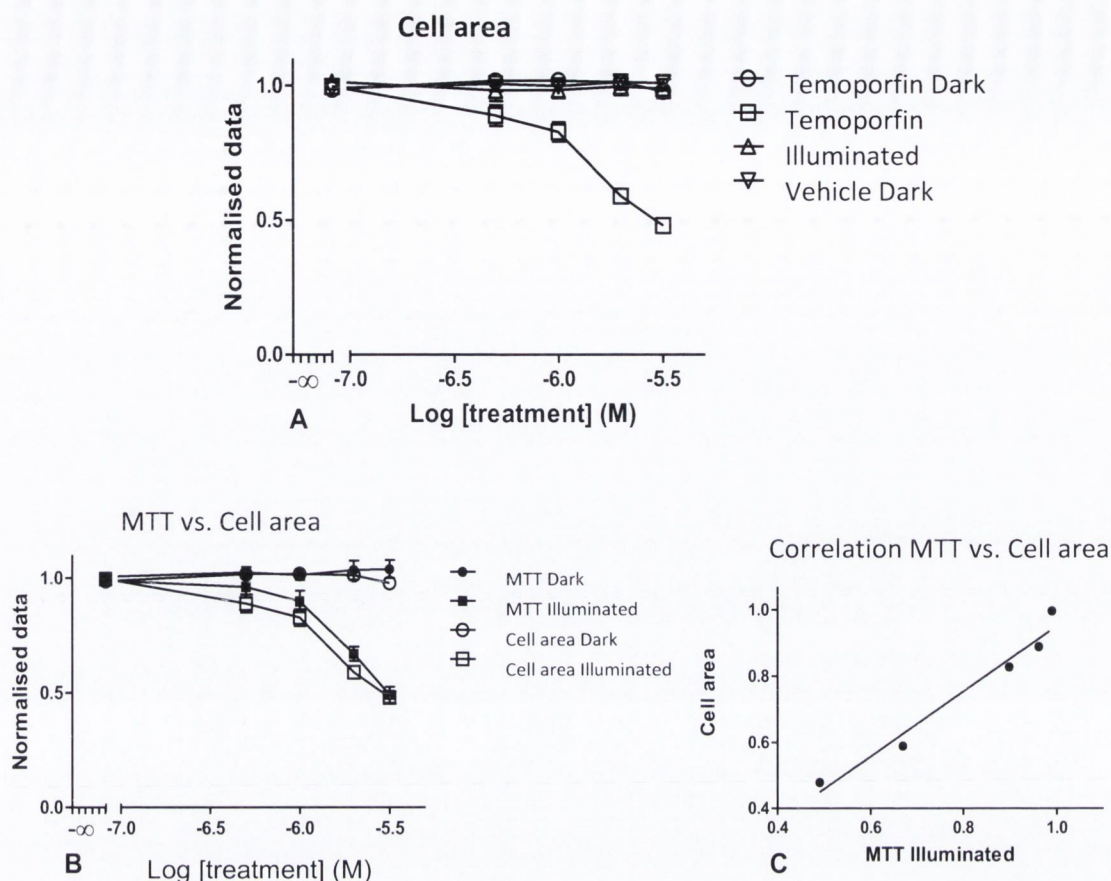


Figure 3.2.2.4 Graphical representation of the cell area data generated using the InCell image analysis (A and B) using OE21 cells. Representation of the cell area with increasing concentrations of Temoporfin and with equivalent concentrations of vehicle illuminated and dark cells (A). Data generated by MTT were compared to InCell data (B). The data generated for illuminated cells were compared in order to establish the degree of correlation (C). Data are representative of four independent experiments and values are expressed in mean $\pm$ SEM.

SKGT-4 cultures exhibited the same trend (Fig. 3.2.2.5 A) and the ANOVA test for a comparison of illuminated and dark cell area showed a significant difference in the data up to, but not including, the 1 μ M dose. A comparison of the MTT in the dark with cell area in the dark showed no statistically significant difference in the data across all concentrations tested. But once again, when comparing their illuminated counterparts a significant difference in the data at 1 μ M was found. This particular parameter showed the same trend as the classical MTT assay and is as sensitive in detecting toxicity (Fig. 3.2.2.5 B). A Pearson

correlation test showed a high correlation between the MTT results and the HCA cell area with $P=0.00258$ with a 99 % confidence interval from 0.49 to 0.99 (Fig. 3.2.2.5 C).

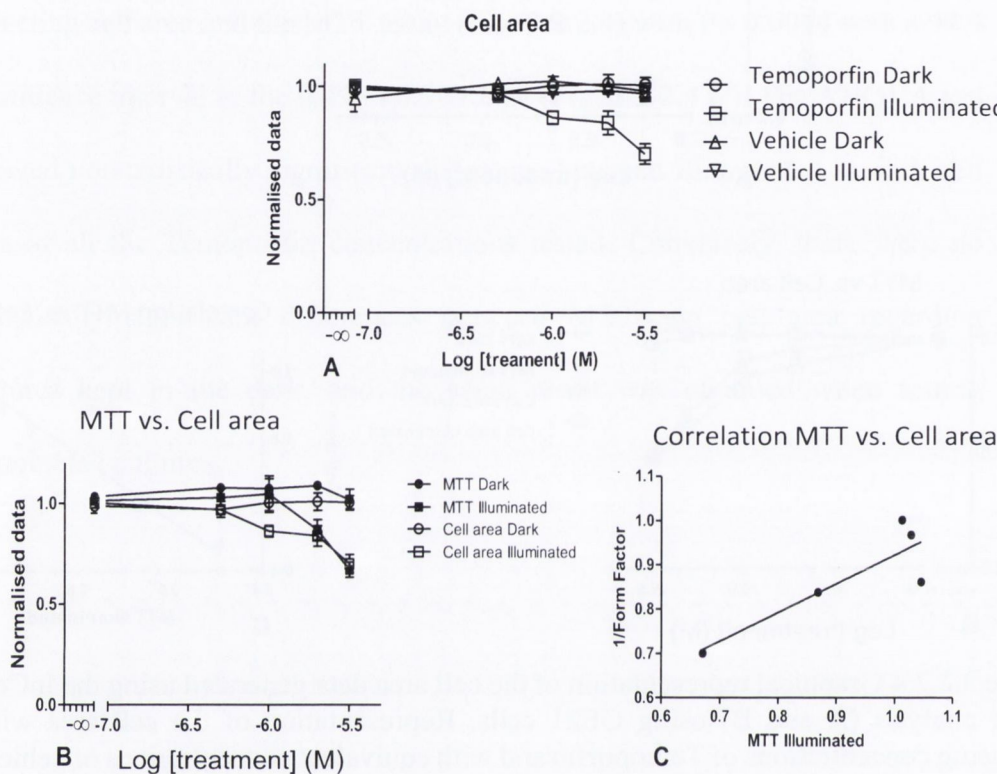


Figure 3.2.2.5 Graphical representation of the cell area data generated using the InCell image analysis (A) using SKGT-4 cells. Representation of the cell area with increasing concentrations of Temoporfin and with equivalent concentrations of vehicle (A) illuminated and dark cells (A). Data generated by MTT were compared to InCell data (B). The data generated for illuminated cells were compared in order to establish the degree of correlation (C). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM

Other morphological parameters were investigated, yet failed to yield any informative data. Among these were nuclear displacement, shape, roundness and cell elongation, and intensity spreading. While the images obtained qualitatively indicated that these parameters could reflect toxicity this could not be confirmed in a statistically reliable manner. All measurements parameters indicate that 2

μM of *m*THPC is sufficient to give a PDT response, as it is the first concentration to give a significant difference between cultures kept in the dark and those that were illuminated in OE21 cells. However, as the range of concentrations used did not give at least 50% inhibition of proliferation in SKGT-4 cells, hence further studies were performed with OE21 cells alone.

A significant advantage of using PDT related drugs with HCA is the inherent fluorescence of the drugs. While this is essential for the desired intra- and extracellular action it also provides an extremely valuable information tool. Compared to the HCA of non-fluorescent drugs no staining or processing of the cultures is needed to access information. For example, as shown in Fig. 3.2.2.6 and Fig. 3.2.2.7, PS fluorescence can be used to monitor photosensitiser uptake. Again, using the HCA platform two parameters were utilized for the analysis: normalised intracellular intensity (Fig. 3.2.2.6 A, 3.2.2.6 C and 3.2.2.7 A) and area of the photosensitiser (Fig. 3.2.2.6 B, 6D and 3.2.2.7 B).

Intensity measurements showed that there is an increase in intensity with increased concentrations of Temoporfin and this was not significantly affected by illuminating the culture (Fig. 3.2.2.6 A and 3.2.2.6 C) in both cell lines tested. This was assessed by means of student t-test where $P = 0.75$ with 99% confidence interval of -208.5 to 253.8 for OE21 cells and $P = 0.91$ with 99% confidence interval of -106.7 to 114.7 for SKGT-4 cells. Linear regressions fitted to the OE21 data showed that in both illuminated and dark cultures there was a significant deviation from zero, with $r^2 = 0.79$ with $P < 0.0001$ for dark cultures and $r^2 = 0.72$ with $P < 0.0001$ for illuminated cultures (Fig. 3.2.2.6 A). Linear regressions fitted to the SKGT-4 data revealed that for both illuminated and dark cultures there was a significant deviation from zero, with $r^2 = 0.56$ with P

=0.0011 for dark cultures and $r^2 = 0.89$ with $P < 0.0001$ for illuminated cultures (Fig. 3.2.2.6 C).

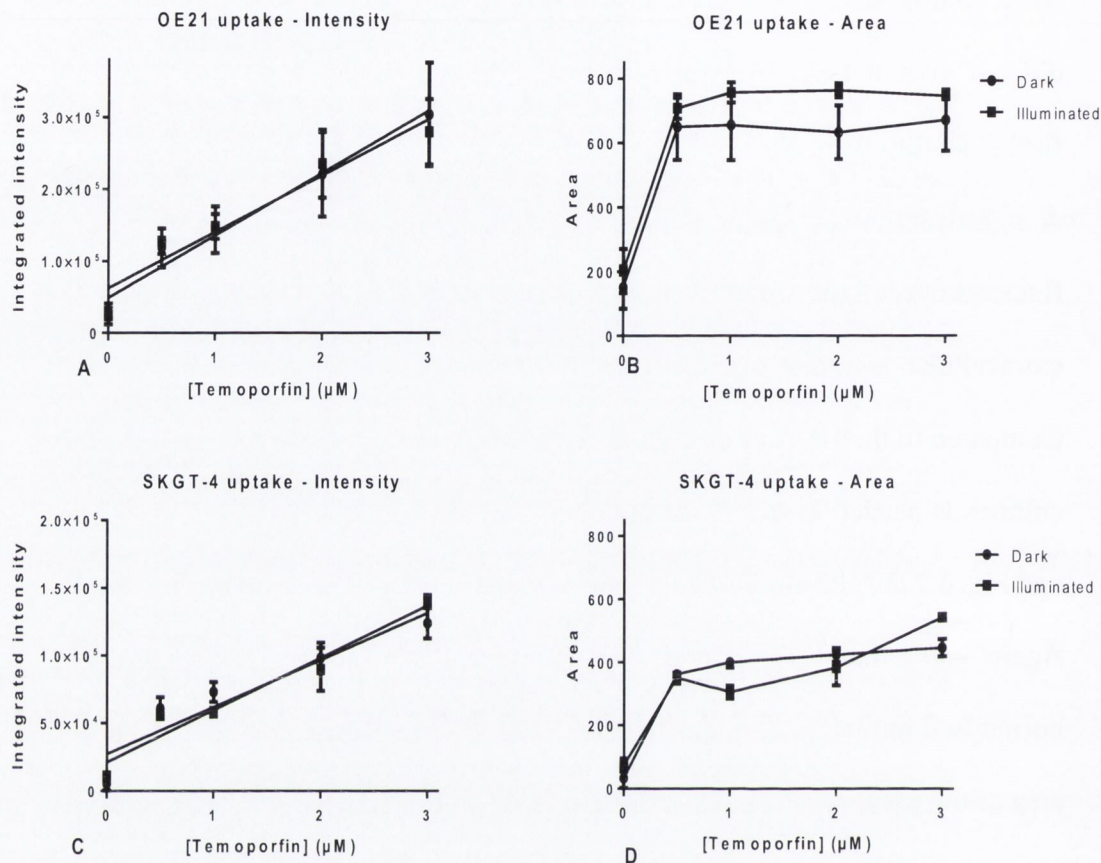


Figure 3.2.2.6 Graphical representation of the results obtained from InCell analysis for photosensitiser parameters. Intracellular intensity (A) and area (B) in OE21 cells; Intracellular intensity (C) and area (D) in SKGT-4 cells. Data are representative of three independent experiments and values are expressed in mean ± SEM.

Note, that the intensity of the PS appears to be much higher in OE21 cells than in SKGT-4. This may indicate that the PS is more concentrated in OE21 cells and this can account for the fact that we were unable to inhibit at least 50% of growth in SKGT-4 cells. For OE21 cells, the Temoporfin intracellular area exhibited a linear increase of area within the cell up to 1 μM of dosage; any higher doses did not yield a higher area within the cells (Fig. 3.2.2.6 B). This suggests that Temoporfin is occupying a finite area within the cell, as even though the area occupied by Temoporfin stabilizes the fluorescence intensity

continues to increase. This is in agreement with published results on the localization of the drug in the Golgi apparatus and the endoplasmic reticulum [182, 186, 187]. The same pattern was seen in SKGT-4 cultures (Fig. 3.2.2.6 D). However, here illumination appeared to alter the area that Temoporfin occupies in the cells. Nevertheless, a t-test showed that illumination causes no significant difference to Temoporfin area in any of the cell lines tested (Fig. 3.2.2.6 B and 3.2.2.6 D); OE21 cells gave a $P = 0.75$ with a 99% confidence interval of -208.5 to 253.8 and SKGT-4 cells $P = 0.99$ with a 99% confidence interval of 357.5 to 361.3.

Using 2 μM of Temoporfin in OE21 cells, its uptake was tracked with time for up to 24 hours (Fig. 3.2.2.7). Following the fluorescence intensity with time revealed a linear increase in intensity up to 24 hours of incubation (Fig. 3.2.2.7A). The non-linear regression (exponential) fitted to the data showed a significant deviation from zero with $r^2 = 0.94$ (Fig. 3.2.2.7A). This result is in accordance to the literature, where uptake of Temoporfin was found to be optimum at 24 hours for other cell lines [188-190]. Also, an incubation period of 24 hours yielded a higher uptake of Temoporfin than any of the other times tested and thus could yield higher toxic effects. Initially, a linear increase in Temoporfin area from 0 to 5 hours was noted and then the area stabilizes (Fig. 3.2.2.7 B). Thus, cells should be incubated for a minimum time of five hours prior to illumination.

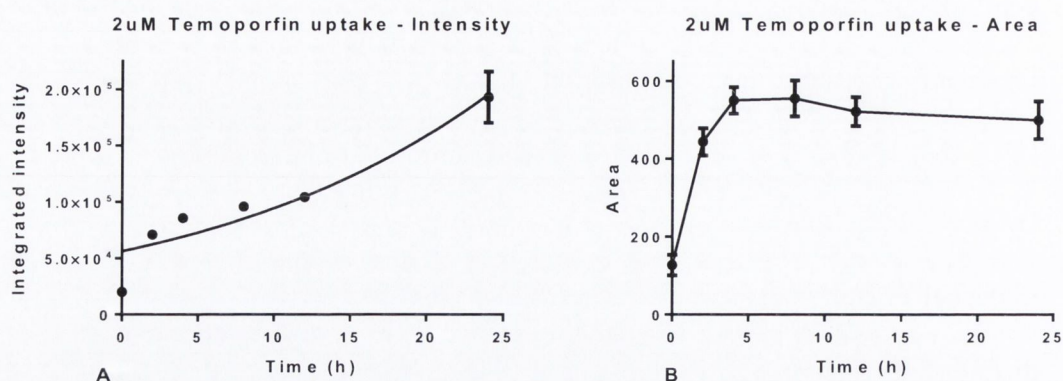


Figure 3.2.2.7 Graphical representation of the results obtained from InCell analysis for photosensitiser parameters in OE21 cells. Intracellular intensity (A) and area of the photosensitiser (B). In this we followed OE21 cultures incubated with Temoporfin for various times. Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

Cell proliferation is directly dependent on cell health and impairment thereof affects the cell number [187, 190, 191]. Thus, monitoring of the cell number over time could, potentially, be an important marker of culture health and, consequently, be used to predict toxicity of possible therapeutics in an *in vitro* assessment. Thus, OE21 cultures were incubated with 2 μ M of Temoporfin, the cell numbers were determined and compared to nuclear and cellular area over the course of 24 hours (Fig. 3.2.2.8). The results show that non-illuminated cells did not exhibit a change in any of the parameters over time (Fig. 3.2.2.8 A and 3.2.2.8 B). On the other hand, cell area decreased in a linear fashion from 0 to 4 hours and then stabilized. As this parameter stabilized the cell number began to decrease significantly from 2 to 24 hours (Fig. 3.2.2.8 A). In the same fashion, nuclear area decreased steadily from 0 to 12 hours and then stabilized. Again, at this time, the cell number continued to decrease in a linear fashion (Fig. 3.2.2.8 B). This clearly illustrates the cell number to be a more reliable marker when tracking a culture over time.

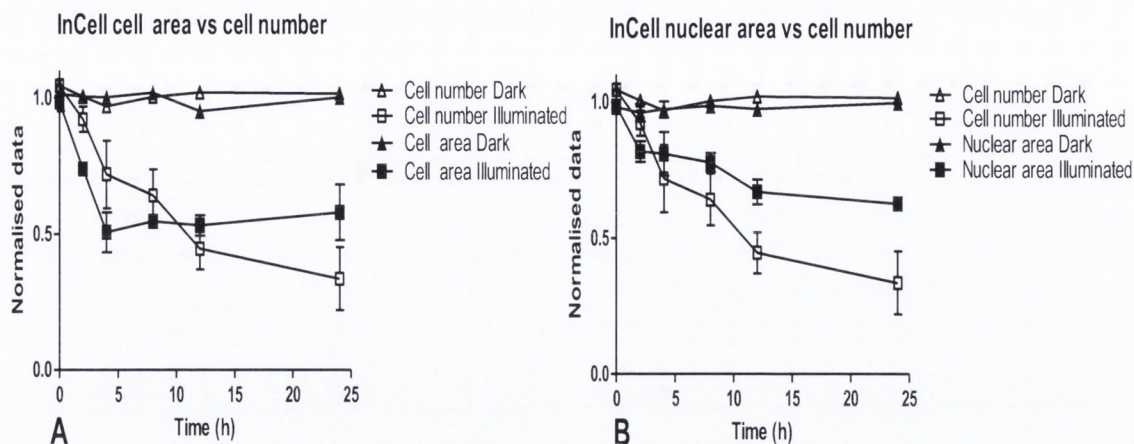


Figure 3.2.2.8 Graphical representation of the results obtained from InCell analysis for OE21 cultures treated with 2 μ M Temoporfin and followed for up to 24 hours. Cell number was compared to cell area over time (A) and to nuclear area over time (B). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

Using HCA we identified several cellular parameters which were validated against the MTT assay. Of these parameters the most robust were nuclear and cellular area in OE21 cells and 1/Form factor and cell area in SKGT-4 cells. In addition, it is possible to obtain detailed information on the concentration dependency of PS in living cellular systems from both intensity and area occupied by Temoporfin within the both types of cell lines tested in a single experiment. The new assay reliably validated that light, vehicle, and Temoporfin alone do not induce cell death and reproduced the difference between illuminated and non-illuminated cells treated with Temoporfin. Similarly, it was possible to follow PS uptake in a time and concentration dependent manner; especially when using the cell number as a measure of toxicity.

3.2 Application of HCS protocol to libraries of porphyrins

3.3.1 Proof of concept: Small library of five porphyrins

In order to further validate our hypothesis, we tested a panel of five porphyrins using both methodologies in OE21 cells using a single concentration of 2 μ M (Fig. 3.2. 10). The five compounds studied were: **1** – 5-(4-methylphenyl)-10-phenyl-15-(2,4,6-trimethoxyphenyl)porphyrin; **2** – *m*THPP [5,10,15,20-tetrakis(3-hydroxyphenyl)porphyrin], the parent porphyrin of *m*THPC; **3** – 5-(4-hydroxyphenyl)-10, 20-diphenyl porphyrin; **4** – 7-dimethyl-7-formyl-chlorin *e*₆; **5** – 5-(4-benzoic acid)-10,20-di(3-methoxyphenyl)porphyrin (Figure 3.3.1.1).

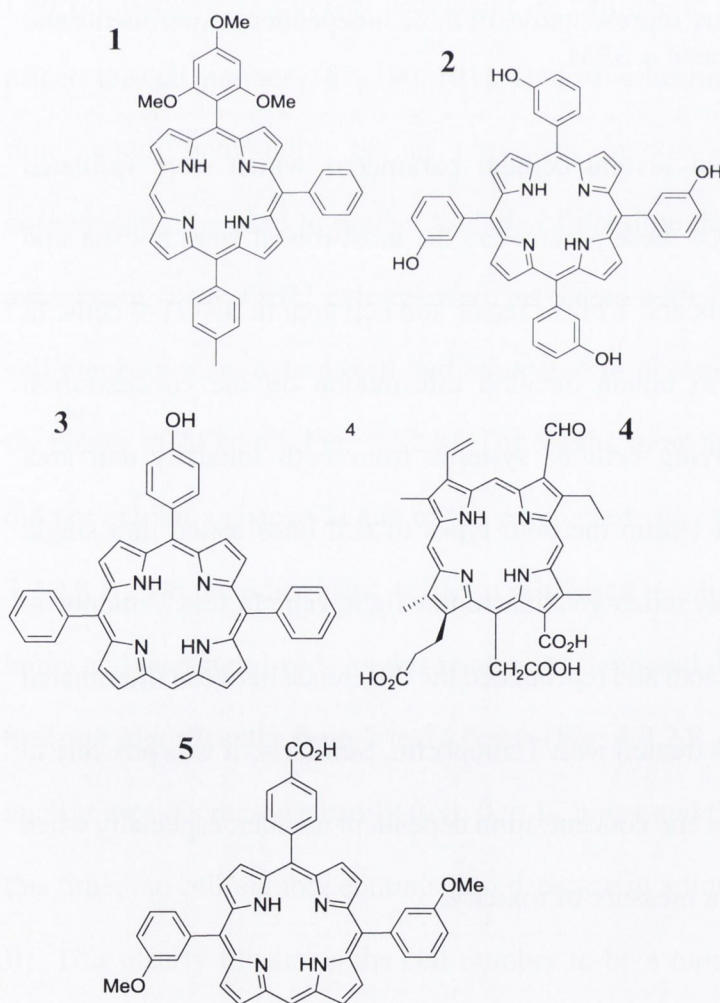


Figure 3.3.1.1 Chemical structures of various compounds tested.

The one way ANOVA results of these experiments with Bonferroni's Multiple Comparison test showed no difference between any of the vehicles tested (either illuminated or in the dark); only compounds **2** and **4** showed any effect upon illumination (Fig. 3.3.1.2). Thus, dose-response studies (both MTT and HCA) were performed with these hits. The results were visually confirmed from the HCA images. These show that, in fact only cultures treated with Temoporfin and **2** and **4** and subsequently illuminated showed any morphological changes (Fig. 3.3.1.3).

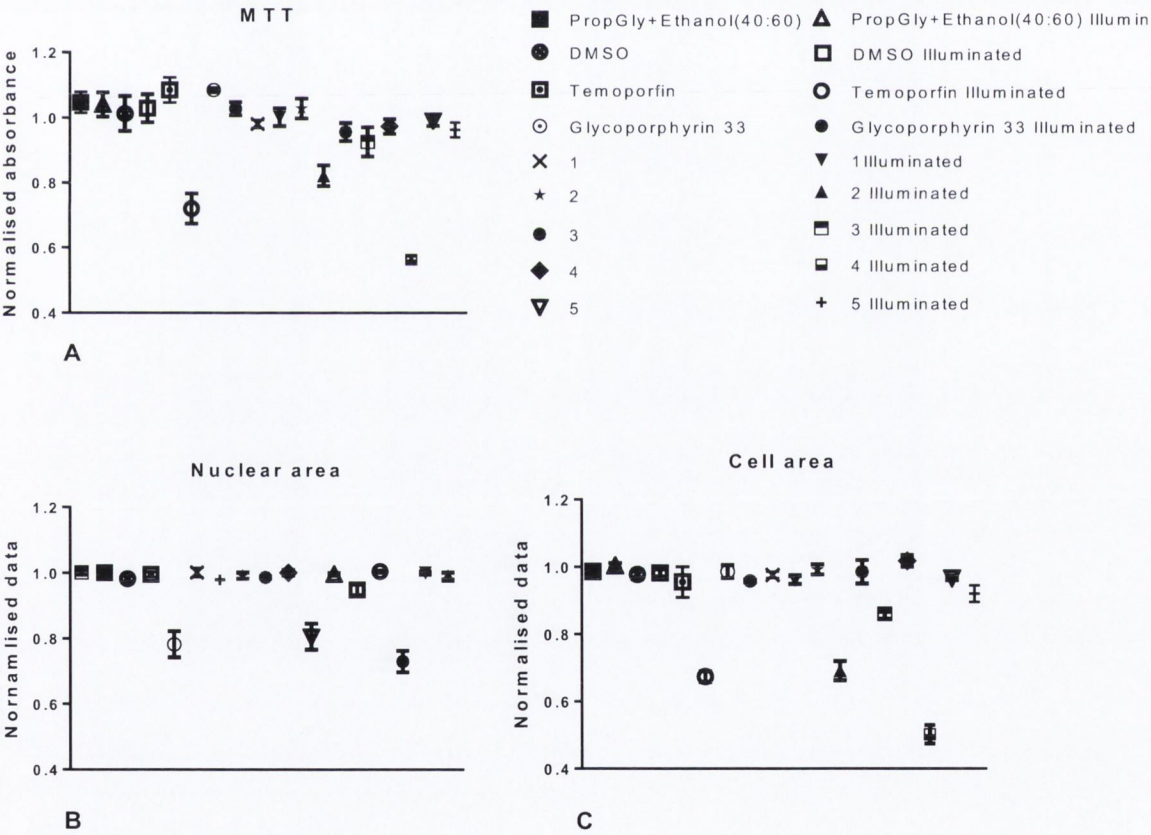


Figure 3.3.1.2 Graphical representation of five porphyrins and respective controls (vehicle controls – Prop+Et / DMSO; positive control – Temoporfin and negative control – glycoporphyrin 33 (5,10 di- β -glucose-porphyrinato zinc(II)) at 2 μ M concentration. MTT data (A) was compared with the previously selected HCA parameters, nuclear area (B) and cell area (C). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM

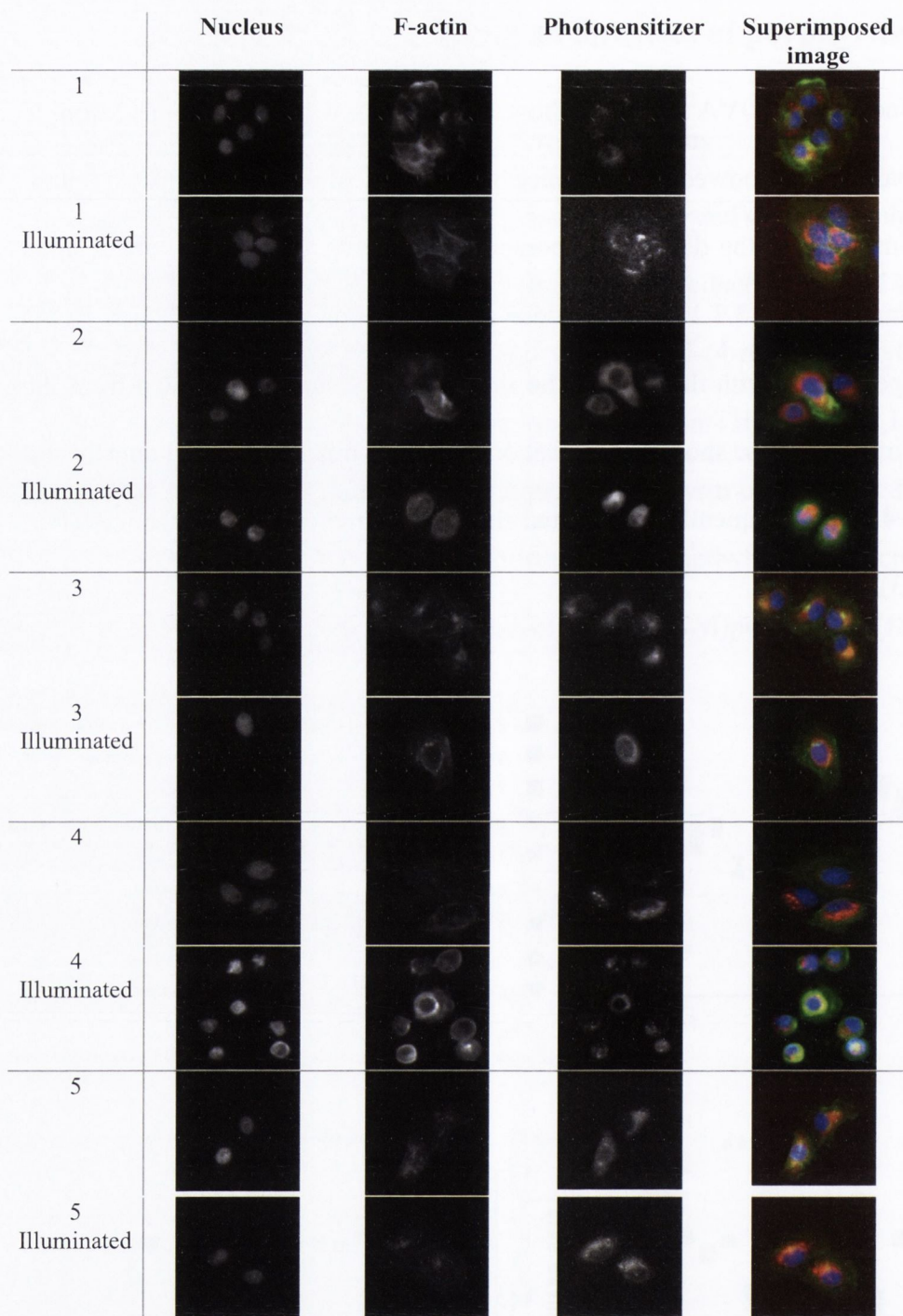


Figure 3.3.1.3 Examples of images collected from the InCell imaging system for the different porphyrins tested in OE21 cells at a single concentration of 2 μ M. The images represents the Hoechst stained nucleus, phalloidin stained F-actin and photosensitiser.

We also tested the vehicles used in all concentrations for toxicity (Fig. 3.3.1.4) using MTT (Fig. 3.3.1.4 A) and HCA parameters established here (Fig. 3.3.14 B and 3.3.1.4 C). Our results showed that the vehicles used showed no toxicity at

any of the concentrations used (Fig. 3.3.1.4) and the HCA parameters gave the same pattern. The two-way ANOVA analysis confirmed these results throughout both methods.

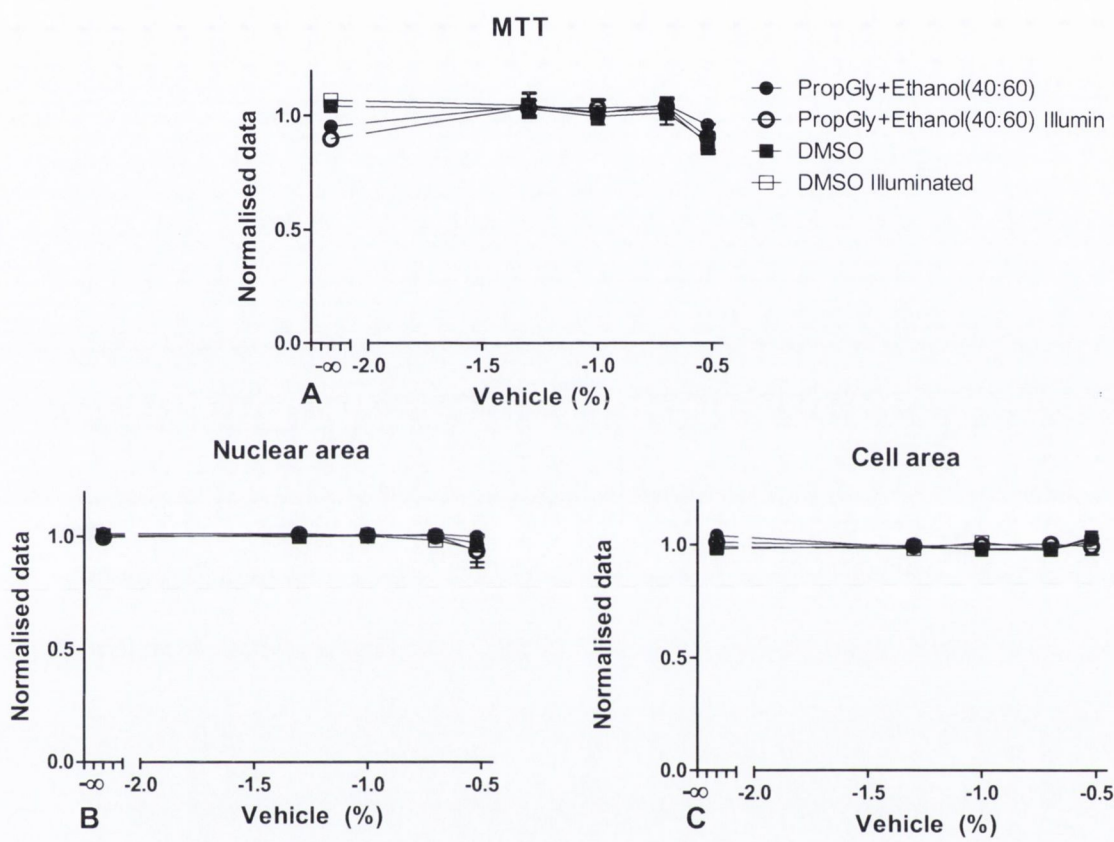


Figure 3.3.1.4 Graphical representation of the results obtained for the test of the vehicles used in all dose-response experiments. MTT data (A) was compared to nuclear area (B) and cell area (C). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

Next we proceeded to test various concentrations of the porphyrin **2** (Fig. 3.3.1.5), and compared the results to Temoporfin as positive control, and to a non-phototoxic glycoporphyrin as negative control. The HCA method was used, and validated by MTT, to determine the toxicity of **2** within the previously tested concentration range (Fig. 3.3.1.5). The results show that **2** followed a similar pattern of action as Temoporfin. This is not too surprising as **2** is a porphyrin analogue of the Temoporfin PS [192]. Overall, the MTT evaluation of **2** (Fig. 3.3.1.5 A) confirmed the data from the HCA study. No differences were found

between **2** and Temoporfin in the dark; for the illuminated cells a difference was only found at 3 μ M alone. Similar results were found for the nuclear area (Fig. 3.3.1.5 B) and a high correlation between nuclear area and MTT data was shown by a Pearson correlation; with $P < 0.0001$ with a 99 % confidence interval from 0.95 to 1 (Fig. 3.3.1.5 D).

The cell area plot (Fig. 3.3.1.5 C) showed significant differences only when comparing Temoporfin treated cells kept in the dark to those that were illuminated from, but not including 0.5 μ M. Similar results were obtained with cells treated with **2** in the dark and compared to illuminated ones. The results suggest that cell area is a more sensitive parameter at detecting toxicity. Conversely, there were no statistically significant differences between any of the other parameters tested. The correlation of the HCA data with the MTT assay was, again, significant with $P < 0.0001$ with a 99 % confidence interval from 0.96 to 1 (Fig. 3.3.1.5 E).

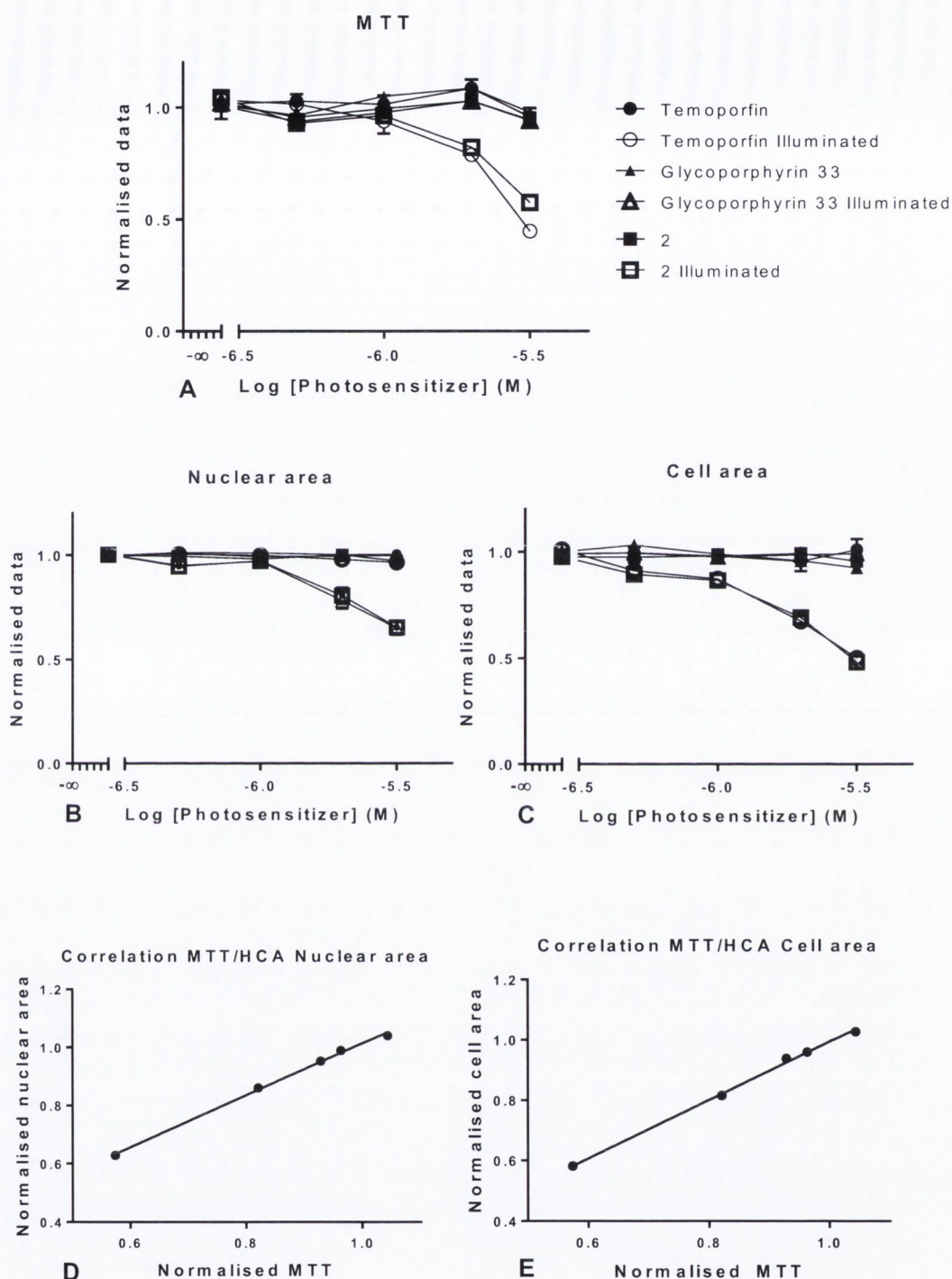


Figure 3.3.1.5 Graphical representation of the data for porphyrin 2, using MTT (A) and the InCell image analysis (B and C) in OE21 cells. Representation of the nuclear area with increasing concentrations of Temoporfin and porphyrin 2 (B) and the equivalent for cellular area (C) in illuminated and dark cells (A and B). Data for illuminated cells were compared to establish the degree of correlation with both HCA parameters tested (D and E). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM and linear regressions were fitted (A, B and C).

The initial screening data in OE21 cells indicated that compound **4** exhibited a more powerful inhibitory effect than the positive control, Temoporfin (Fig. 3.3.1.6 A). As a rhodochlorin derivative this agrees with expectations, as such systems have been shown to be highly suitable PS [193]. The MTT evaluation of **4** (Fig. 3.3.1.6 A) showed a statistically significant difference between Temoporfin treated cultures and those treated with **4** at 2 at 0.5 μ M concentrations. A detailed analysis of cells treated with **4** and those treated and illuminated showed significant difference at all concentrations tested. In fact, a comparison of illuminated Temoporfin cells *versus* **4** illuminated cultures gave statistically significant differences throughout all concentrations tested (Fig. 3.3.1.6 A). Thus, **4** has a stronger inhibitory effect than Temoporfin against OE21 cells. All other results were as expected; no difference was found between dark and illuminated cultures treated with the control, non-phototoxic glycoporphyrin (5,10 di- β -glucose-porphyrinato zinc(II)) [194].

Similarly, overall the same results were obtained for the nuclear area (Fig. 3.3.1.6 B) and a high correlation between nuclear area and MTT data was confirmed by a Pearson correlation with $P=0.0057$ with a 99 % confidence interval from 0.29 to 0.99 (Fig. 3.2.1.6 D). Similar to the studies with **2**, cell area upon treatment with **4** was found to reflect toxicity in a reliable manner. In this case, the correlation of the HCA data with MTT was again significant with $P=0.017$ with a 99 % confidence interval from 0.61 to 0.99 (Fig. 3.3.1.6 E).

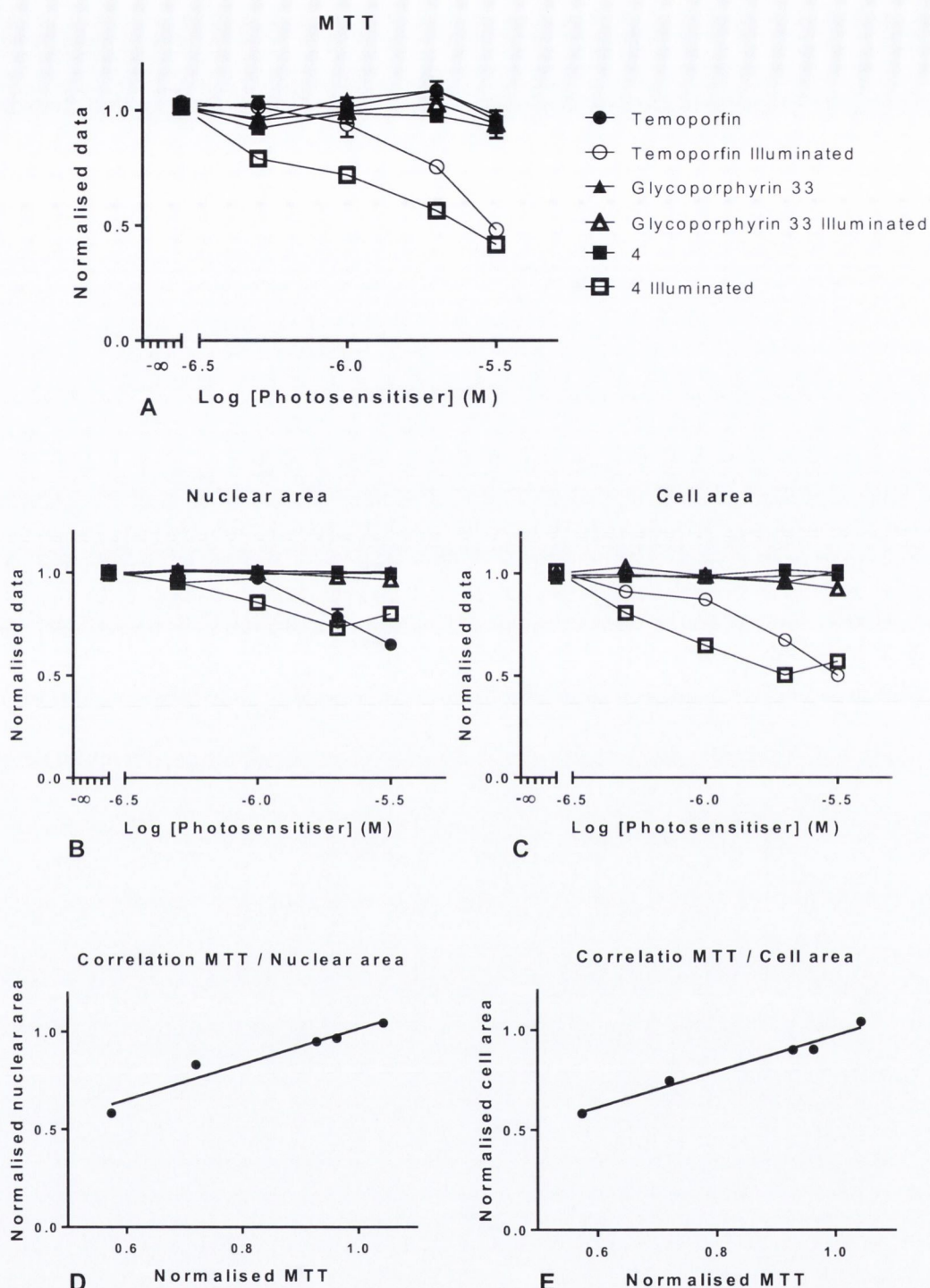


Figure 3.3.1.6 Graphical representation of the data generated for porphyrin 4, using MTT (A) and the InCell image analysis (B and C) in OE21 cells. Representation of the nuclear area with increasing concentrations of Temoporfin and chlorin 4 (B) and the equivalent for cellular area (C) in illuminated and dark cells (A and B). Data generated for illuminated cells were compared to establish the degree of correlation with both HCA parameters tested (D and E). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM and linear regressions were fitted (A, B and C).

As shown above, intensity measurements gave an increase in intensity with increasing concentrations of Temoporfin (Fig. 3.3.1.7 A). This was confirmed for both test compounds (Fig. 3.3.1.7 A). ANOVA analysis revealed **2** to be different from Temoporfin at 3 μ M while **4** differed significantly at 2 and 3 μ M. This can be visually confirmed by the graphical representation where it was observed that **2** has a slightly higher intensity than Temoporfin, probably due to less fluorescent quenching of this compound in the cells. Compound **4** on the other hand showed less fluorescence than Temoporfin in the cells. In Temoporfin treated cells, intracellular area exhibited the expected linear increase of area within the cell up to 1 μ M of dosage (Fig. 3.3.1.7 B) and the same pattern was observed for **2**. For **4** a linear relationship with increasing concentrations was found. However, the area occupied by this compound in the cells treated with 2 and 3 μ M was the same as in Temoporfin treated cells, as validated by ANOVA.

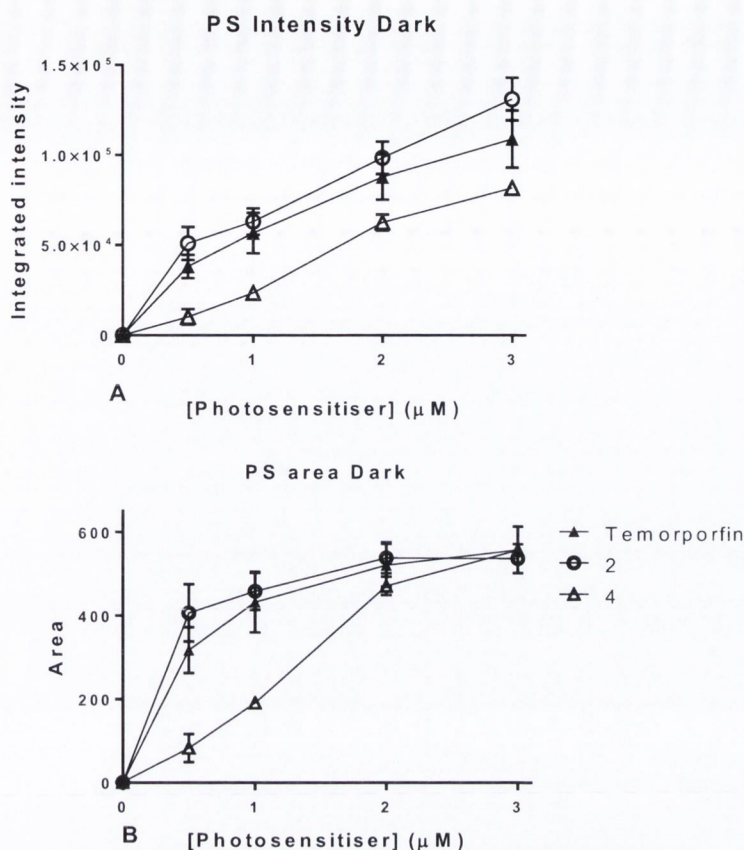


Figure 3.3.1.7 Graphical representation of the results obtained from InCell analysis for photosensitizer parameters in OE21 cells. Intracellular intensity (A) and area of the photosensitizer (B). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

3.3.2 Library of twenty two porphyrins

A new screen of 22 compounds was performed in three cell lines (OE21, SKGT-4 and OE33) using a single concentration of 2 μ M. In order to evaluate our analytical strategy for comparative biological evaluation we decided to investigate a series of porphyrin (or derivatives) photosensitisers with a varied degree of photoactivity. The compounds have been divided into the three sub-groups consisting of hemes, pheophorbides, porphyrins and ABCD-type structures [195, 196].

The compounds analyzed were: **6** – haematoporphyrin; **7** – hemin; **8** – methyl pheophorbide b; **9** – glucose pentaacetate; **10** – 5-(3,6-dimethoxyphenyl)-

2,3,7,8,12,13,17,18-octaethyl-10,15,20-triphenylporphyrin; **11** – 5-bromo-15-hexyl-10,20-bis(3-hydroxyphenyl)porphyrin; **12** – (E)-3-[(5,10,15-triphenylporphyrinato-20-yl)nickel(II)]acrylic acid; **13** – 3-hydroxymethyl-4-nitrophenol; **14** – (2-(2-chloroethyl)-17-ethyl-3,7,13,18-tetramethyl-12-methoxycarbonyl-8-(propionic acid methyl ester)porphyrinato)zinc(II); **15** – methyl pheophorbide a; **16** – (E)-methyl 4-(5,10,15-triphenylporphyrin-20-yl)but-2-enoate; **17** – (2E,2'E)-dimethyl 4,4'-[5,15-triphenylporphyrin-10,20-diyl]dibut-2-enoate; **18** – 5-(4-benzoic acid)-15-hexyl-10,20-bis(3-hydroxyphenyl)porphyrin; **19** – methyl 5-(4-benzoic acid)-15-hexyl-10,20-bis(3-hydroxyphenyl)porphyrin; **20** – 5-(2-cyanoethyl)-10,15,20-tris(4-methylphenyl)porphyrin; **21** – (5,10,15-triphenylporphyrinato)zinc(II); **22** – (2-(2-chloroethyl)-17-ethyl-3,7,13,18-tetramethyl-12-methoxycarbonyl-8-(propionylpiperidine amide)porphyrinato)zinc(II); **23** – 5-(4-ethynylphenyl)-10,20-diphenylporphyrin; **24** – protoporphyrin IX dimethyl ester; **25** – (2,3,7,8,12,13,17,18-octaethyl-5-nitro-porphyrinato)zinc(II); **26** – [5-hexyl-10,20-diphenyl-15-(spirobis-1,3-dithian-2-yl)porphyrinato]nickel(II); **27** – chlorin e6 trimethyl ester (Fig. 3.3.2.1).

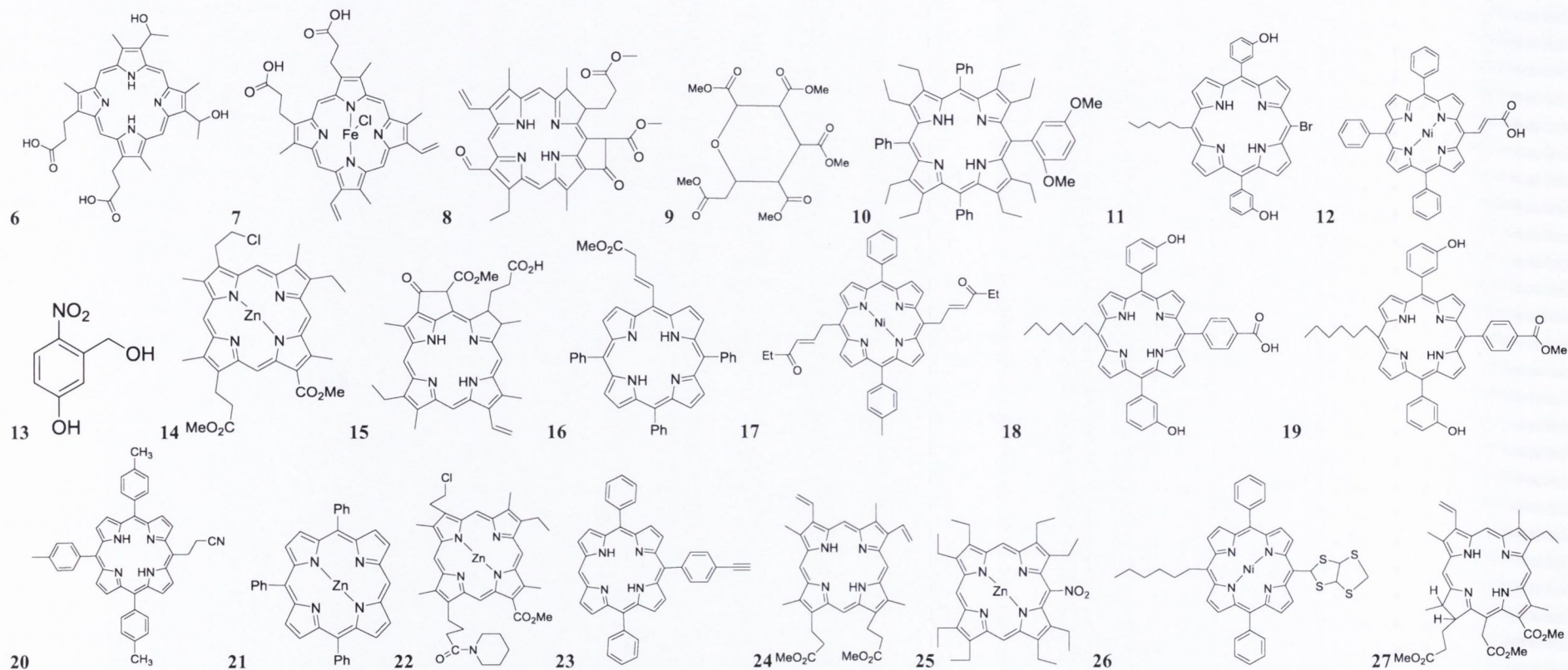


Figure 3.3.2.1 Chemical structures of all compounds tested representing a wide scope of porphyrin and derivatives.

From these experiments it was determined that non-illuminated PS treated cultures showed no toxicity (data not shown). However, when cultures were illuminated we found that only compounds **8**, **15**, **24** and **27** showed activity in all cell lines (Fig. 3.3.2.2). Nuclear area, however, once again failed to reveal any relevant toxicity information in SKGT-4 cells as it mostly remained unchanged (data not show). Nonetheless, the large number of output parameters from the image analysis indicated that the parameter 1/form factor can yield significant information about the toxicity of *m*THPC and the test compounds in SKGT-4 cells. This cell line differs much in appearance to OE21 and OE33 cells. The results showed that the 1/form factor in SKGT-4 (Fig. 3.3.2.2 C) cells in fact reflects the phototoxicity of Temoporfin and compounds **8**, **15**, **24** and **27** in a similar fashion as does the nuclear area for OE21 (Figure 3.3.2.2 A) and OE33 cells (Fig. 3.3.2.2 B). No toxicity was observed upon treatment with vehicle alone (Fig. 3.3.2.2 A, B and C). These results were confirmed by the cell area analysis for all three analysed cell lines (Fig. 3.3.2.2 D, E and F). Of note is the fact that different cell lines seem to respond differently to the different compounds tested.

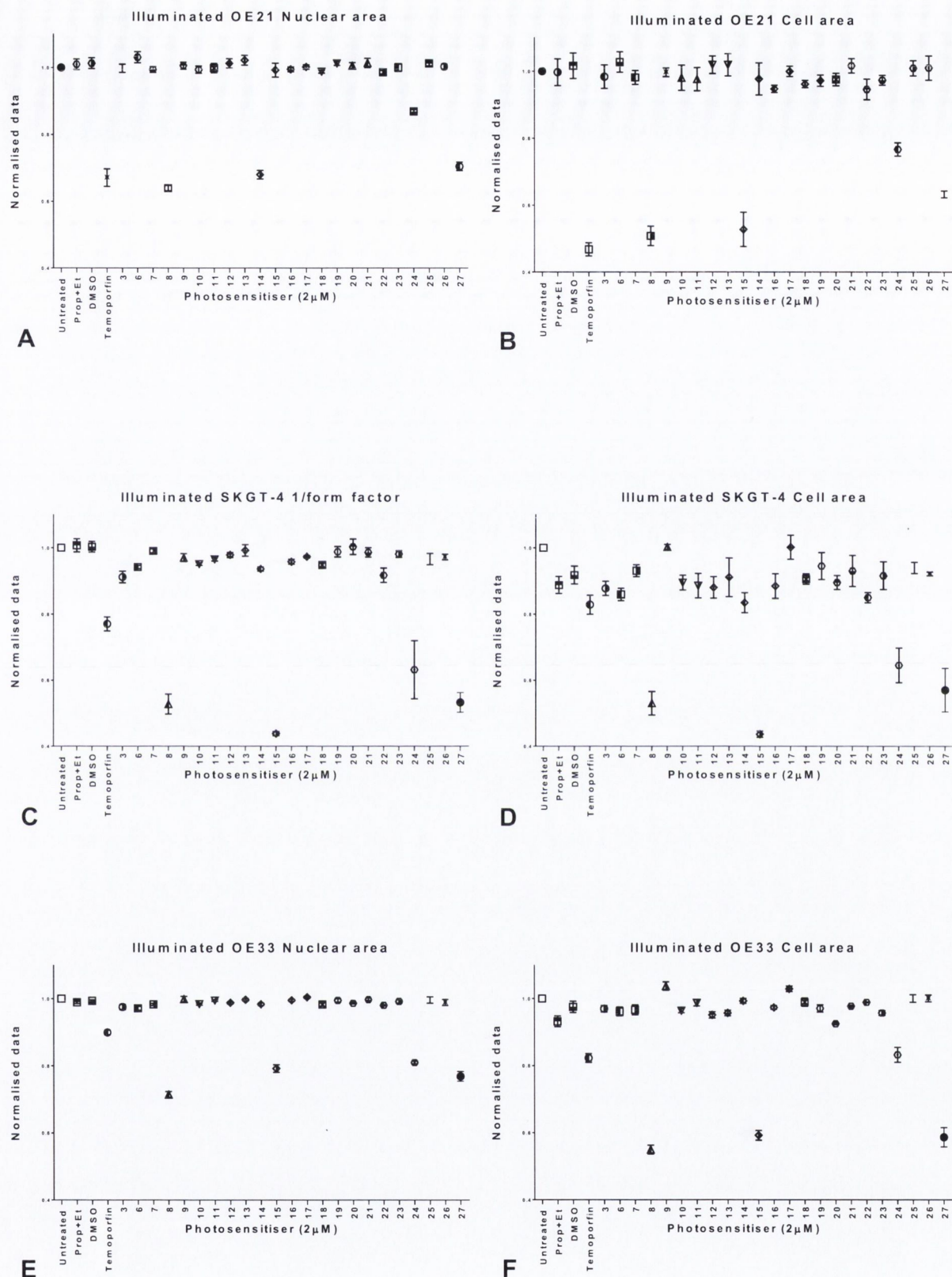


Figure 3.3.2.2 Graphical representation of the results obtained from InCell analysis for a library of 22 different compounds tested in OE21 cells (A) OE33 cells (B) and SKGT-4 cells (C). In terms of nuclear area (A and B) and 1/form factor (C) and cell area (D, E and F). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

From the compound parameters (integrated intensity and area) it was determined that from the 22 compounds tested only **8**, **14**, **15**, **18**, **22**, **24** and **27** were taken up by the cells (Fig. 3.3.2.3 C, D and E), this explains many of the results obtained for the activity output, in fact most compounds are not taken up by the cells at all. Results showed that compounds **8**, **15**, **18** and **24** (Fig. 3.3.2.2 A, B and C) occupy a similar intracellular space as *m*THPC, but compounds **14**, **22** and **27** occupy a different area of the cells (Fig. 3.3.2.3 A, B and C). The previously discussed trend where integrated intensity seems to be directly related to effectiveness of the compound is maintained in this larger primary screen (Fig. 3.3.2.2 and Fig. 3.3.2.3 C, D and E).

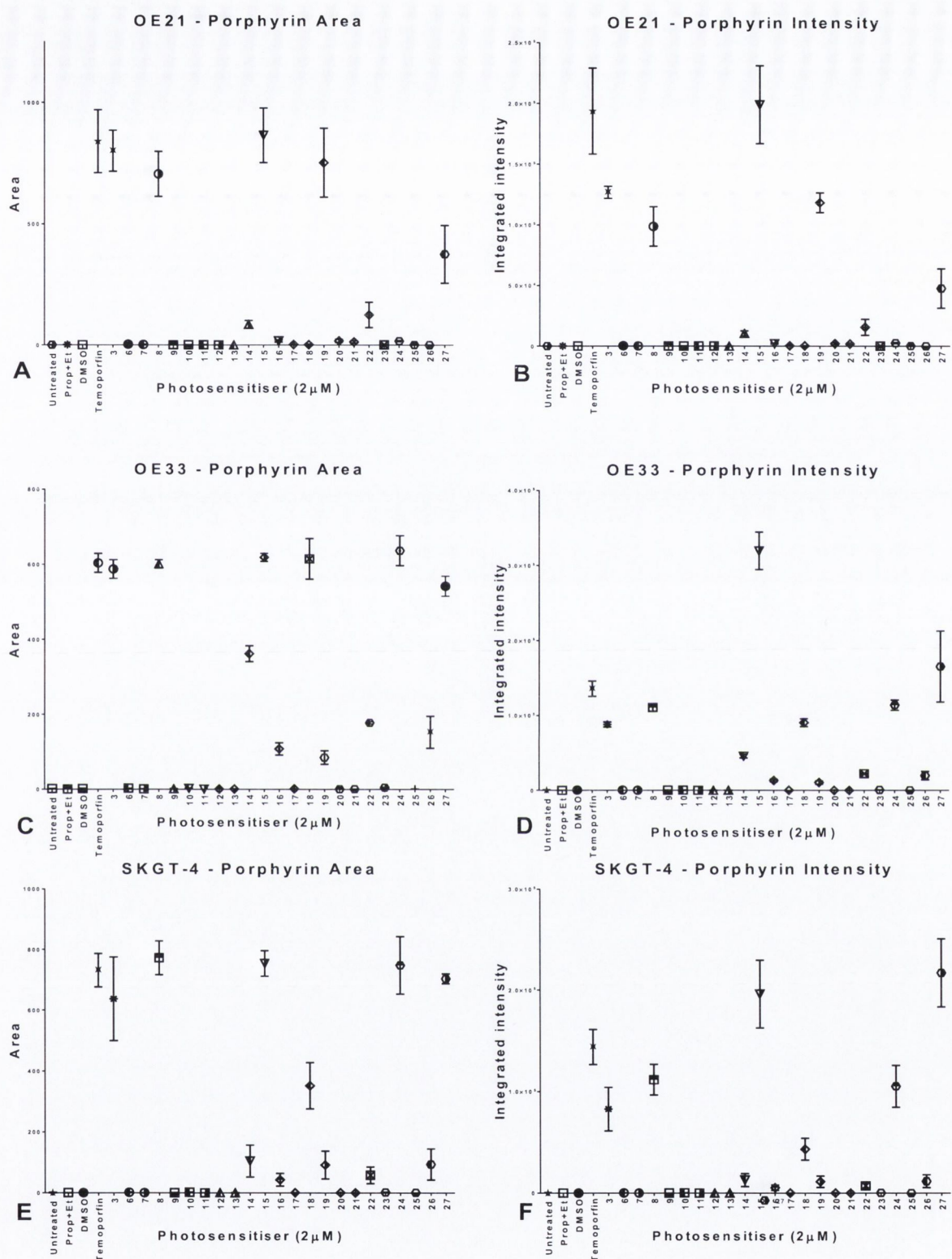


Figure 3.3.2.3 Graphical representation of the results obtained from InCell analysis for a library of 22 different compounds tested in OE21 cells (A) OE33 cells (B) and SKGT-4 cells (C). In terms of integrated intensity of the potential photosensitisers (A, B and C) and area (D, E and F). Data are representative of three independent experiments and values are expressed in mean ± SEM.

The identified hits (compounds from both screens) were then analysed by MTS assay at a final post illumination time of 24 hours; this not only further validated our early time point of 4 h but also allowed us to determine IC₅₀ values for each of the hits identified in the previous screens. From these results we were able to determine that the various cell lines do, in fact, respond to photodynamic treatments differently both in illuminated and dark conditions (Fig. 3.3.2.4). We were also able to ascertain that the results obtained at 4 hours are representative of a longer post illumination time as the IC₅₀ values determined for OE21 cells remained unchanged (Fig. 3.3.2.1). The same could be inferred for the other cell lines. Of note is the fact that the chosen concentration range showed the beginning of dark toxicity at the higher 24 µM, allowing us to further compare the compounds analysed.

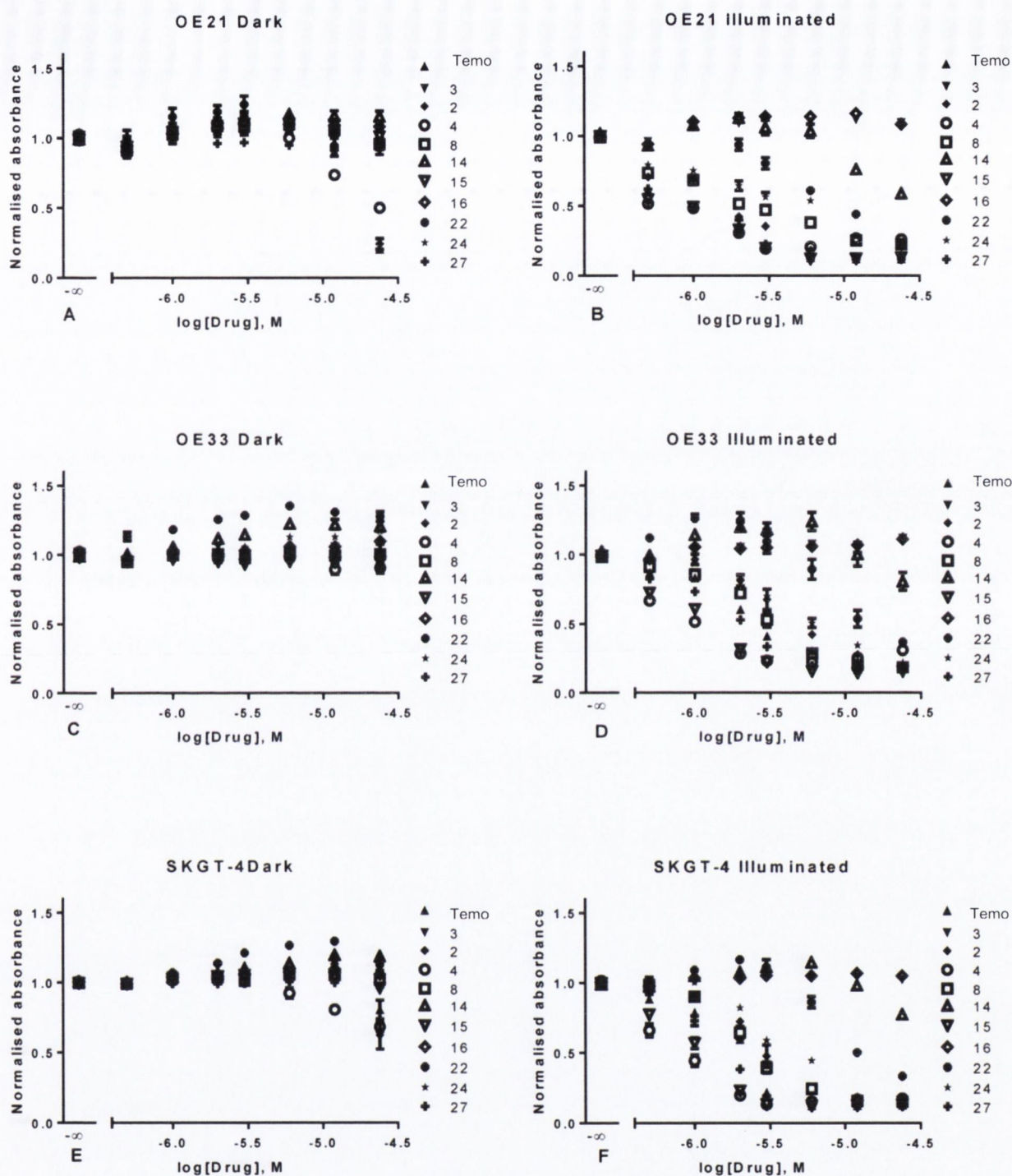


Figure 3.3.2.4 Graphical representation of the results obtained from the 24 h MTS assay performed in the previously identified hits (compounds **2**, **4**, **8**, **15**, **22**, **24** and **27**) as well as three non-toxic compounds (**3**, **14** and **16**) tested in OE21 cells (A and B) OE33 cells (C and D) and SKGT-4 cells (E and F). Data are representative of three independent experiments and values are expressed in mean ± SEM.

The results confirmed that at this concentration OE21 cells are more susceptible to dark toxicity from Temoporfin and compound **2** whereas compound **4** showed less response than the positive control (Fig. 3.3.2.4 A). No such dark toxicity was seen for OE33 cells making them much more resistant to these types of compounds (Fig. 3.3.2.4 C). The SKGT-4 cell line showed strong dark toxicity for Temoporfin, **2** and **4** with no significant difference between any of the three compounds (Fig. 3.3.2.4 E).

In addition, the results confirm the lack of toxicity of compounds **3**, **14** and **16**; if an IC₅₀ could be determined the r² values are too low in order for these to be deemed statistically valid (Table 3.3.2.1). These were considered to be an example of compounds that tested non-toxic in the primary screen. In fact, all compounds that were deemed to be non-toxic but were taken up by the cells could be used as negative controls for further experiments.

Table 3.3.2.1 IC₅₀ values determined for the various compounds screened and cells lines tested when illuminated.

	Temoporfin	3	2	4	8	14	15	16	22	24	27
OE21											
IC50 (μM)	2	5	2	2	2	1	2	7	7	3	2
r ²	0.9	0.3	0.9	0.8	0.9	0.4	0.9	0.5	0.9	0.9	0.9
OE33											
IC50 (μM)	2	Interrupted	4	1	3	1	2	Interrupted	8	5	2
r ²	0.9		0.9	0.9	0.9	0.4	0.9		0.8	0.9	0.9
SKGT-4											
IC50 (μM)	2	Interrupted	3	1	2	Ambiguous	1	Interrupted	8	4	2
r ²	0.9		0.9	0.91	0.9		0.9		0.9	0.9	0.9

These were generated by fitting a non-linear regression (log(inhibitor) versus response with variable slope in Graphpad prism. Results are representative of three independent experiments and values were rounded to units.

In summary, the results herein presented allowed the development and validation of an improved methodology for drug primary screens. The information obtained

using this methodology allows the evaluation of several biologic parameters, such as nuclear and cellular area. Furthermore, the time needed to evaluate the effects the many chemical compounds developed by chemical synthesis is greatly reduced.

3.3.3 Library of novel porphyrins linked to anti-inflammatory molecules

Non – steroidal anti-inflammatories drugs (NSAID) are molecules commonly and widely used in the clinic to control and attenuate many inflammatory conditions commonly resulting from infection [197]. They are used to reduce pain, swelling and / or fever [197]. This class of pharmaceuticals act by inhibiting (reversibly or permanently) the enzyme cyclooxygenase (COX) 1 or 2 (Fig. 3.3.3.1)

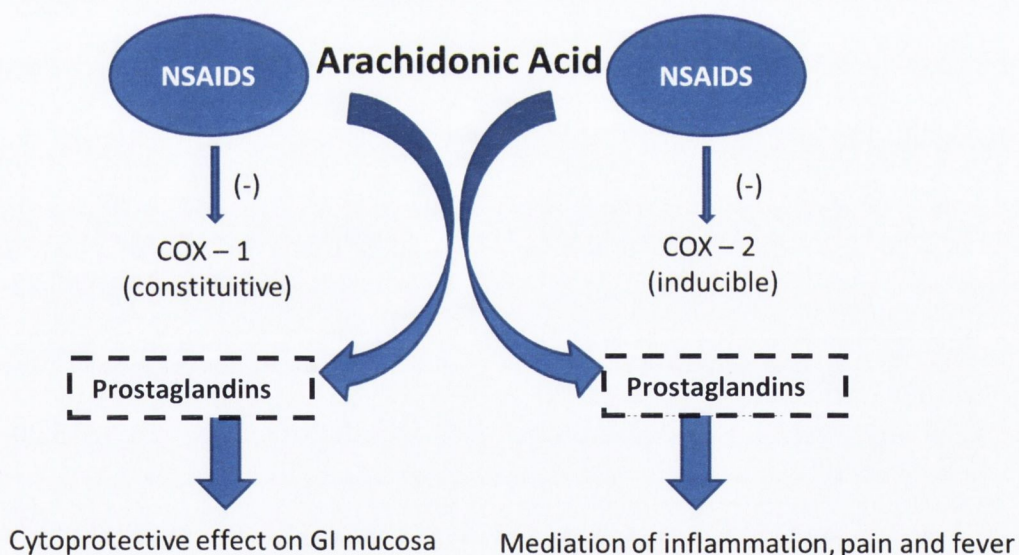


Figure 3.3.3.1 Schematic representation of the mode of action of NSAID; NSAID-induced GI injury can be prevented by sparing the COX-1 constitutive enzyme of the mucosa, by the use of COX-2 selective inhibitors that specifically block the inducible COX-2 isoform expressed at sites of inflammation.

In cancer treatment a fine line between apoptosis and necrosis of cells belonging to tumour tissues and immunity against tumour antigens must be obtained in order

to achieve successful long-term tumour destruction and control [25]. An intervention and commitment from the immune system is essential in increasing tumour destruction as well as destruction of any other cells derived from the initial tumour, even in distant locations of the body [22]. Most of the commonly used cancer treatment are immunosuppressant [22] as they are toxic to the bone marrow (where most of the immunoeffective cells are generated) [22] PDT on the other hand generates three main types of cell death (Figure 3.3.3.2) necrosis, autophagy and apoptosis [198] where necrosis plays a predominant role [199]. Therefore it is important to control the immune response following PDT treatments [200].

Ibuprofen is a NSAID, it is a non-specific COX inhibitor and it remains one of the most commonly used drugs both clinically as well as over the counter, it is very effective in the reduction of inflammation and has the added advantage of being photoactive [201]; it comes to reason that a construct of this anti-inflammatory drug attached to a porphyrin would be ideal as a potential cancer treatment. We tested the phototoxic potential of five different ibuprofen-porphyrin constructs (**28** - 5-(3-hydroxyphenyl),10,15,20-tris[3-(2-(phenylamino)benzoate)phenyl]porphyrin; **29** - 5,10,15,20-tetrakis[3-(2-(phenylamino)benzoate)phenyl]porphyrin; **30** - 5,10-di(3-hydroxyphenyl),15,20-di[(3-(4-isobutylphenyl)propanoate)phenyl]porphyrin ; **31** - 5-(3-hydroxyphenyl),10,15,20-tris[(3-(4-isobutylphenyl)propanoate)phenyl]porphyrin; **32** - 5,10,15,20-tetrakis[(3-(4-isobutylphenyl)propanoate)phenyl]porphyrin) (Fig 3.3.3.3) against out *in vitro* oesophageal tumour models using our HCS method we added a variety of illumination times in order to insure activity would be seen. All compounds were tested at 2µM and cells were allowed to recover for 4 hours prior to fixation, further staining, imaging and analysis.

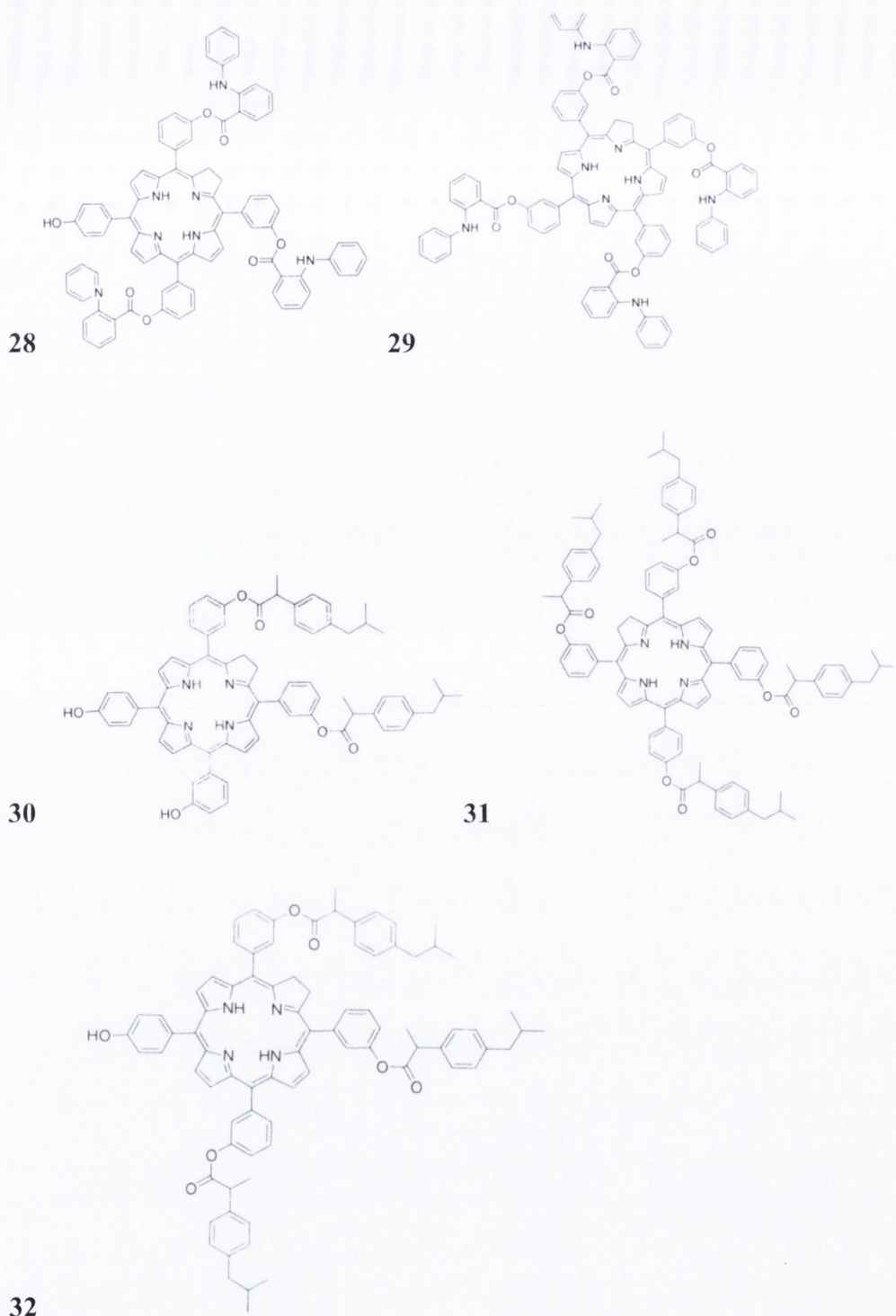


Figure 3.3.3.3 Chemical structure of porphyrins combined with ibuprofen tested using the HCS method previously established.

Our results showed that none of the porphyrin-anti-inflammatories tested had any effect in any of the cell lines tested nor did the illumination times made a

significant difference (Fig. 3.3.3.5 and 3.3.3.6) in the phototoxicity to the cells. In fact, none of the compounds showed significant uptake by the cells (Fig. 3.3.4 and 3.3.7). All of the positive results obtained are, in fact, false positives as the compounds stick to the outside of the cells and the image analysis software accounts for them as being taken up (Fig. 3.3.4 and 3.3.7). This highlighted one of the limitations of our method since the widefield system captured total fluorescence hence there is not a differentiation between fluorescent compounds in the cells and that of external fluorescence, this shortcoming would be easily removed by using a fully confocal high content system. If a widefield systems is used it is essential that an experienced operator visually confirms the results obtained from the automated image collection and analysis.

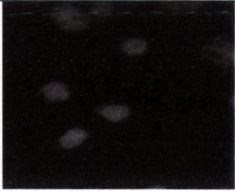
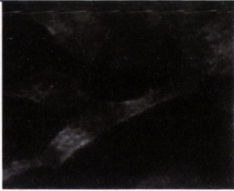
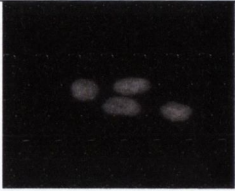
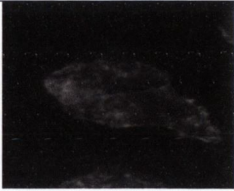
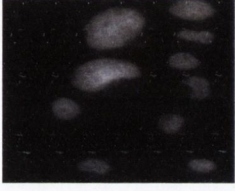
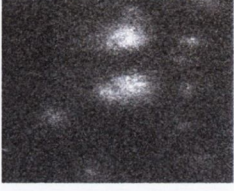
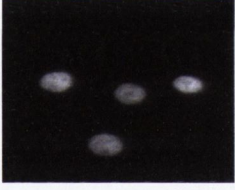
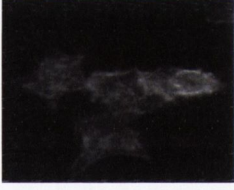
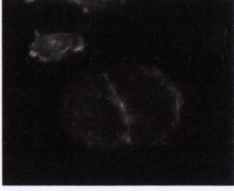
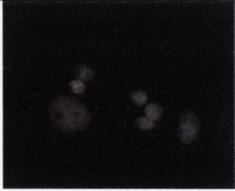
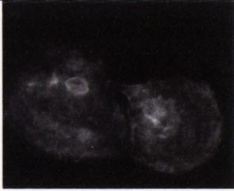
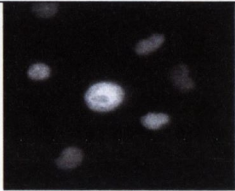
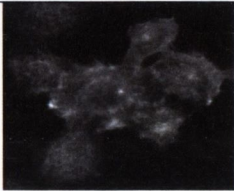
	Nucleus	F-actin	Photosensitiser
Untreated			
Temoporfin			
Screen 2 Porphyrin 14			
28			
29			
30			
31			
32			

Figure 3.3.3.4 Examples of images collected from the InCell imaging system for the different porphyrins tested in SKGT-4 cells at a single concentration of 2 μ M. The images represents the Hoechst stained nucleus, phalloidin stained F-actin and photosensitiser.

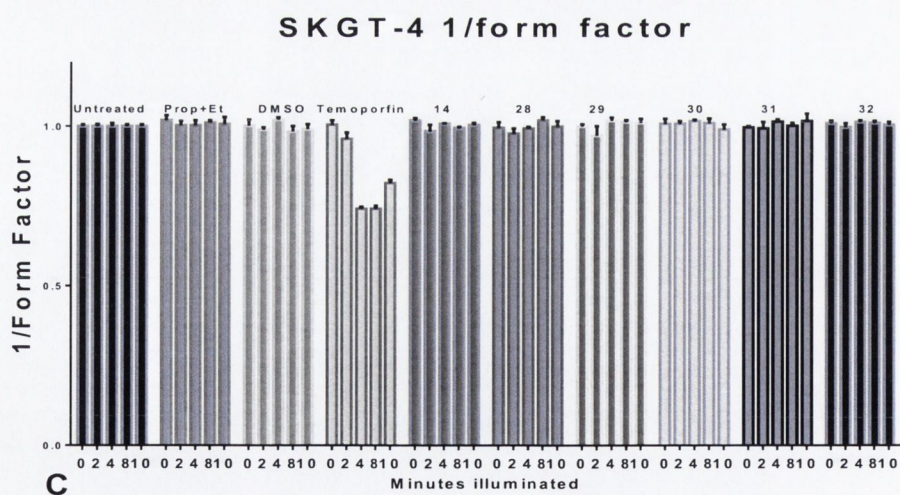
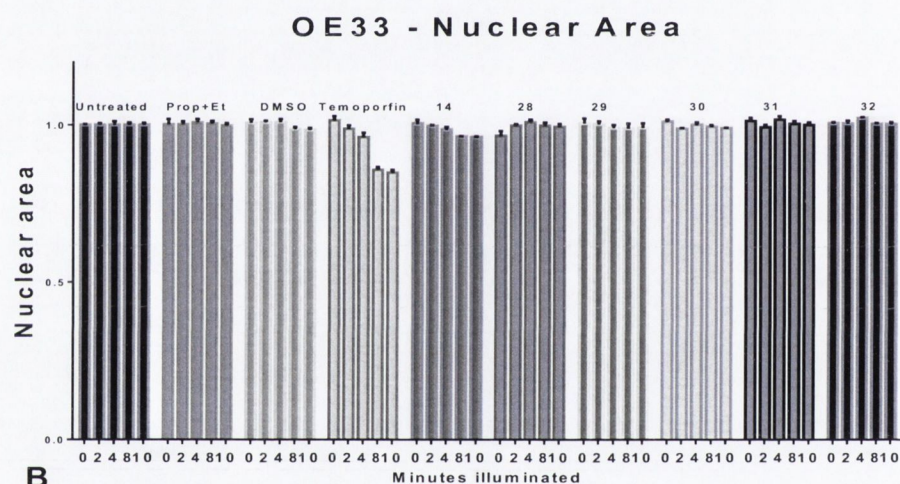
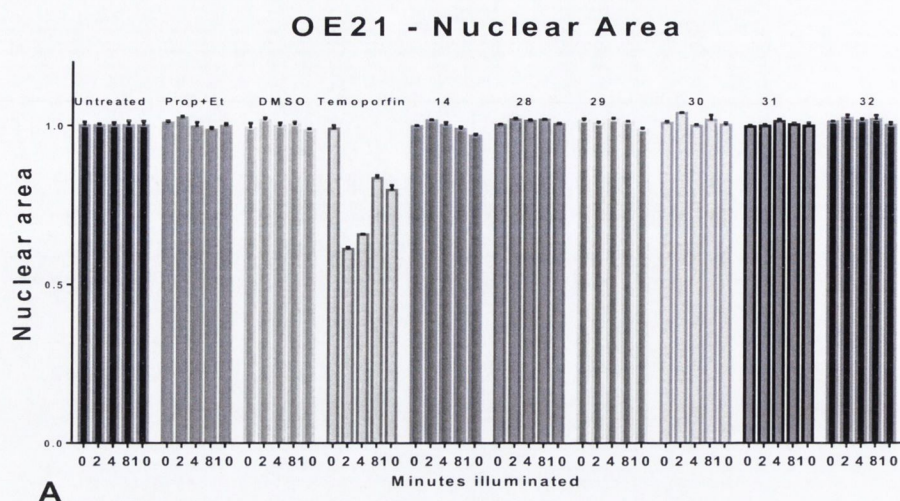


Figure 3.3.3.5 Graphic representation of all porphyrin-antiinflammatory constructs tested in terms of nuclear area (OE21 – A and OE33 – B) and 1/form factor (SKGT-4 – C) and time of illumination.. Data are representative of one experiment and values are expressed in mean $\pm$ SEM.

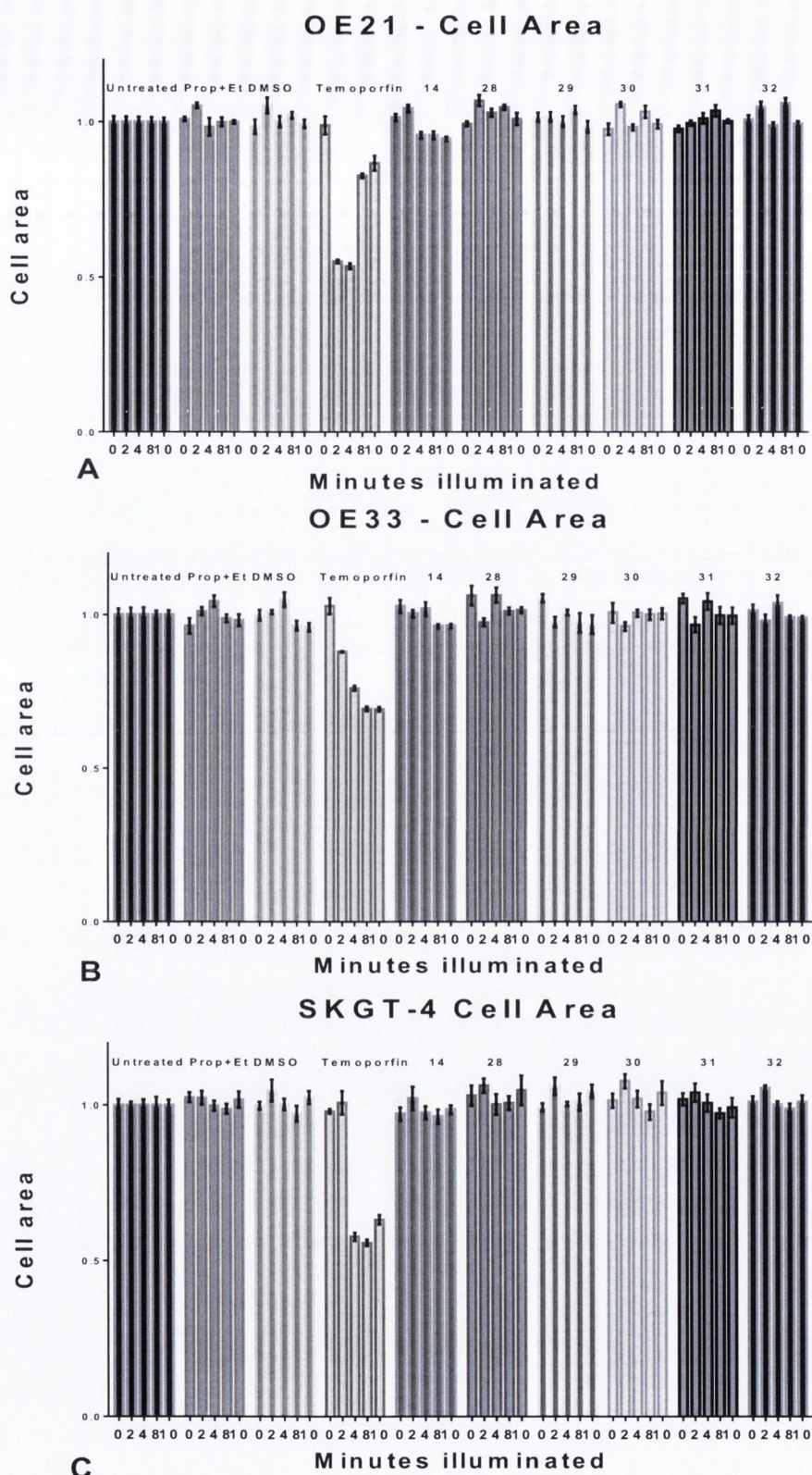


Figure 3.3.3.6 Graphic representation of all porphyrin-ant inflammatory constructs tested in terms of cell area (OE21 – A, OE33 – B and SKGT-4 – C) and time of illumination. Data are representative of one experiment and values are expressed in mean $\pm$ SEM.

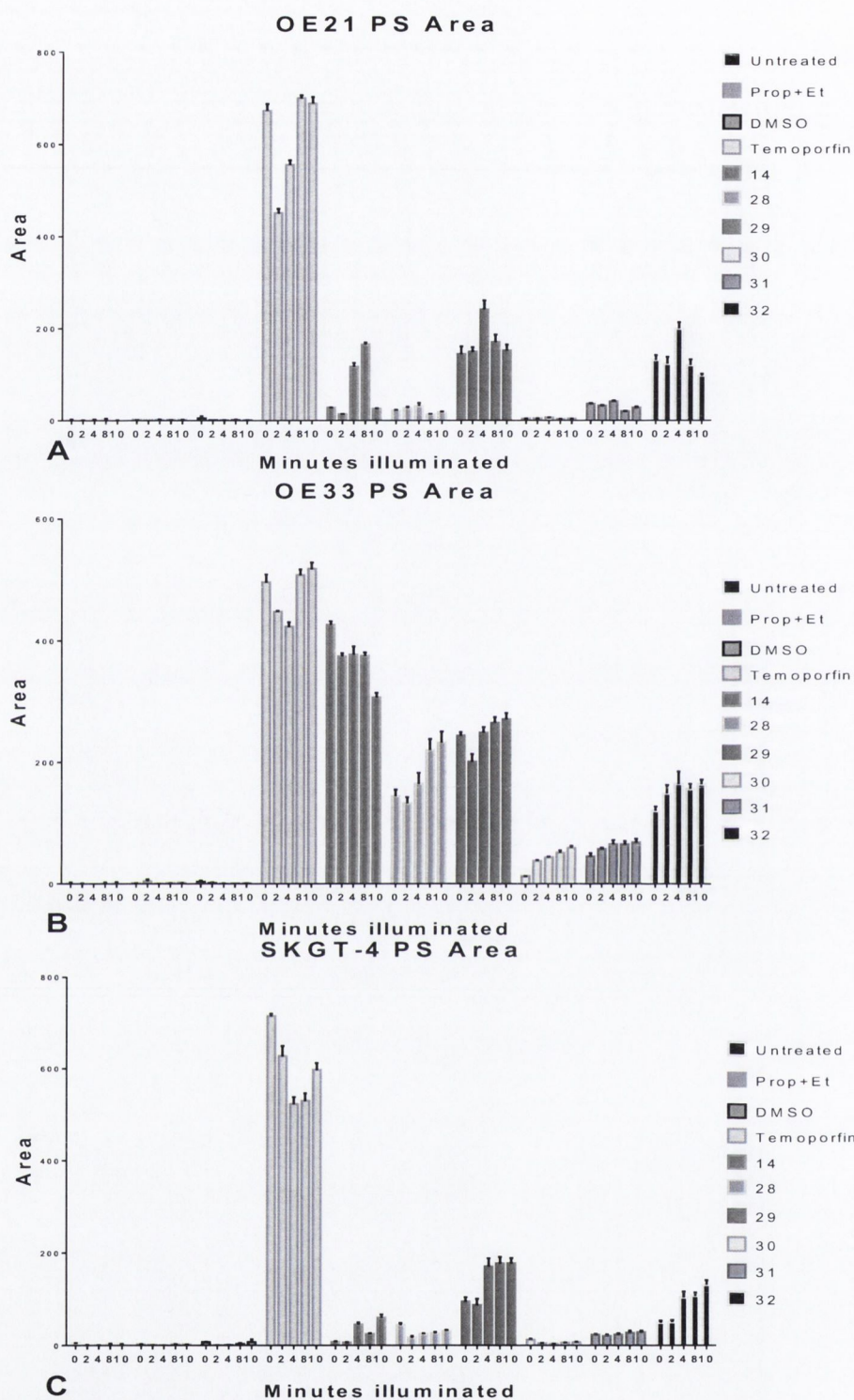


Figure 3.3.3.7 Graphical representation of the results obtained from InCell analysis for photosensitiser intercellular area in OE21 cells (A), OE33 cells (B) and SKGT-4 (C). Data are representative of one experiment and values are expressed in mean $\pm$ SEM.

As none of the compounds tested showed any activity or significant uptake hence no new experiments were performed nor any further graphical assessment done; instead a new chemical synthetic strategy is currently being designed to develop biologically active alternatives in this new class of compounds.

3.3.4 Library of novel glycoporphyrins

Carbohydrates are important biomolecules that play a fundamental role in many biological processes, including fertilization, neuronal development, cell–cell communication, hormone activities and inflammatory responses [202-205]. Synthetic oligosaccharides and glycoconjugates offer fascinating applications for medicinal chemistry as anti-adhesion agents, drug-delivery systems, imaging agents and vaccines [206-209]. Their potent biological activity is in part due to their ability to bind specific host lectins and to modulate host immune response [210-212]. Altered glycosylation is a universal feature of cancer cells and certain glycan structures are well known biomarkers for tumour progression [213-215]. As well as displaying altered glycosylation patterns, the surfaces of cancer cells are known to over-express certain carbohydrate-binding lectins [216-219]. Specific carbohydrate–lectin interactions allow for the direct targeting of glycosylated therapeutics specifically to cancer cells. The ability to target therapeutics to a specific cell type or organ is of significant clinical importance, particularly for cancer treatments such as PDT, [220, 221] in which currently used, clinically approved drugs such as Temoporfin and Photofrin are non-specific and their use can result in the destruction of healthy tissue [194].

Following test of porphyrins, a library of glycosylated porphyrins (glycoporphyrins) was tested. These molecules carry monosaccharides,

synthetic disaccharides and a synthetic trisaccharide. The activity of these compounds against the human oesophageal squamous-cell carcinoma cell line OE21 was performed [194].

In order to assess the potential of the synthetic glycoporphyrins for medical applications, initial *in vitro* studies were performed. Based on our current interest in PDT for oesophageal cancer, we choose the human oesophageal squamous-cell carcinoma cell line OE21 for *in vitro* studies [132]. As a PDT positive control, the well-established, second-generation photosensitiser Temoporfin was used [183, 222]. The glycoporphyrins **33** - 5,10 di- β -glucose-porphyrinato zinc(II); **34** - 5,10 di- α -mannose- porphyrinato zinc(II); **35** - 5,10 di- β -glucosamine- porphyrinato zinc(II); **36** - 5,10 di- β -lactosamine- porphyrinato zinc(II); **37** - 5,15 di- β -mannose- porphyrinato zinc(II); **38** - 5,15 di- β -lactosamine- porphyrinato zinc(II); **39** - 5,10,15-tri- β -glucose- porphyrinato zinc(II); **40** - 5,10, 15 tri- α -mannose- porphyrinato zinc(II); **42** - 5,10,15-tri- β -glucosamine- porphyrinato zinc(II); **42** - 5, 10,15-tri- β -lactosamine- porphyrinato zinc(II); **43** - 5- β -glucosamine-10- α -mannose- porphyrinato zinc(II) (Fig. 3.3.4.1) were used as a representative sample of the present library of compounds for standard MTT assay and HCA analysis. The mono-glycosylated porphyrins were found to be too insoluble for this *in vitro* study.

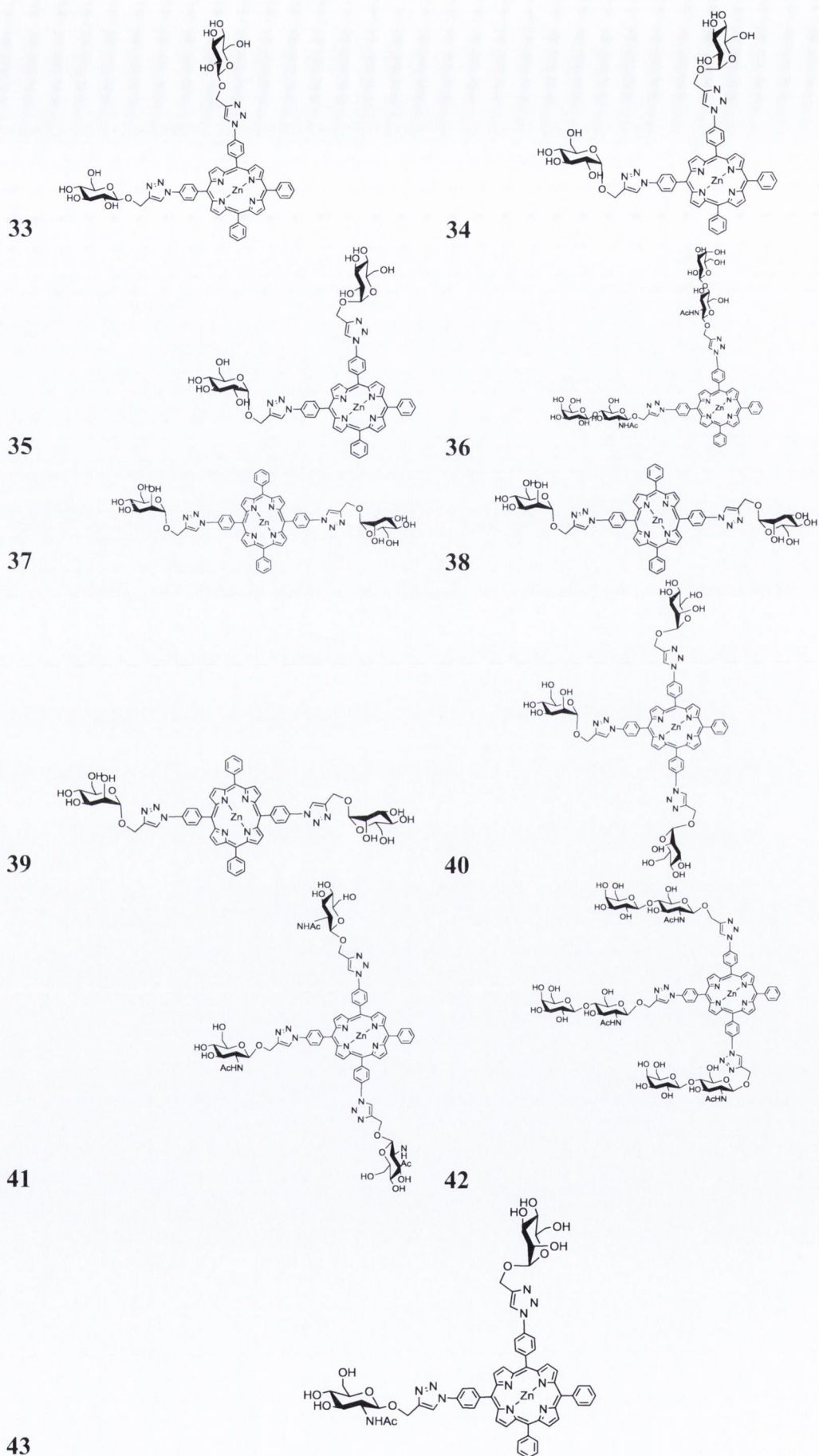


Figure 3.3.4.1 Chemical structure of glycoporphyrins tested.

Upon plate illumination, the MTT assay showed a decrease in cell viability with increasing Temoporfin concentrations (Fig 3.3.4.2), which indicated the validity of the HCA system for PDT. However, no significant decrease was obtained for cells treated with glycoporphyrins at any of the concentrations tested. Results obtained from a two-way ANOVA statistical analysis confirmed that there was no difference between illuminated and dark cultures when treated with any of the eleven glycoporphyrins tested (Table 3.3.4.1). As expected, there was a significant difference in toxicity observed for cells treated with Temoporfin, in which proliferation significantly decreased when the cultures were illuminated (Figure 3.3.4.2). Thus, these glycoporphyrins do not show significant toxicity in the chosen cell line under the experimental conditions used. The photolysis time (2 minutes) was chosen as a clinically relevant timeframe. Based on the photophysical differences, we expected less PDT activity for the glycoporphyrins than for Temoporfin; however, the absence of any activity was surprising as there are many examples in the literature that report a PDT effect of metallic porphyrins in human cells [223-225].

Table 3.3.4.1 Summary of the results obtained from the two way ANOVA statistical analysis. Where X represents no significant difference and √ represents a positive difference between cultures kept in the dark and those that were illuminated.

	33	34	35	36	37	38	39	40	41	42	43
Dark vs Illuminated	X	X	X	X	X	X	X	X	X	X	X
Temoporfin Dark	X	X	X	X	X	X	X	X	X	X	X
Temoporfin Illuminated	√	√	√	√	√	√	√	√	√	√	√

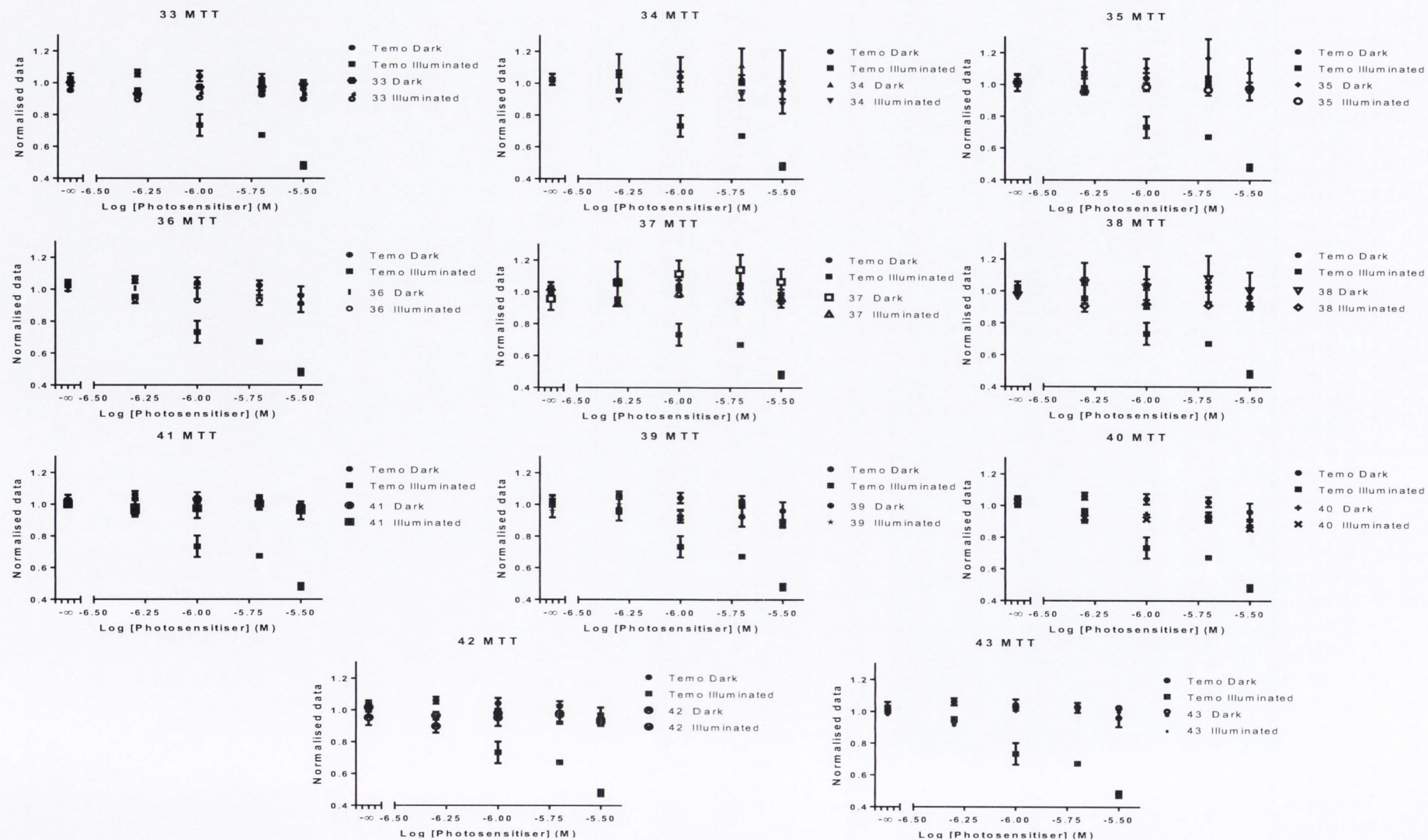


Figure 3.3.4.2 OE21 cells treated with increasing concentrations of Temoporfin and with equivalent concentrations of the test glycoporphyrins (illuminated and non-illuminated); the cultures were assessed for toxicity using a cell proliferation assay (MTT). Data are representative for three independent experiments and values are expressed in mean $\pm$ SEM.

This raised the question of whether the lack of toxicity was due to the compounds being taken up by the cells and then rapidly expelled, or whether the dyes were not taken up at all. Thus, uptake studies were performed. Of the compounds studied, Temoporfin and six glycoporphyrins (**33–37** and **43**) were taken up by OE21 cells. Staining results for these compounds are shown in Fig. 3.3.4.3. It is clear from the images that these glycoporphyrins occupy a finite space within the cytoplasm of the cell. Furthermore, there is evidence that the intracellular fluorescence pattern of these compounds (Fig. 3.3.4.3) is very similar to that exhibited by fluorescence probes targeted to the Golgi apparatus [226-228]. From this, it is possible to infer that the glycoporphyrins are most likely to accumulate in this organelle. This will be investigated further by using co-localization confocal imaging with a commercially available Golgi stain such as Golgi-ID™ (Enzo life sciences, UK) and analysing the images using an automated image analysis.

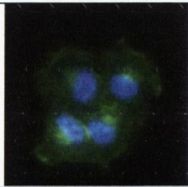
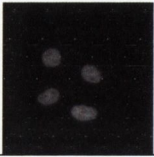
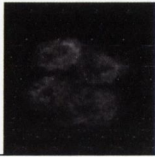
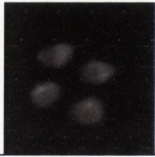
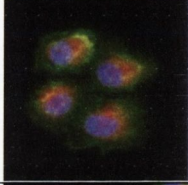
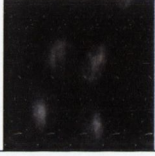
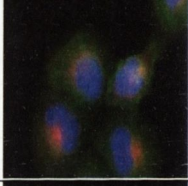
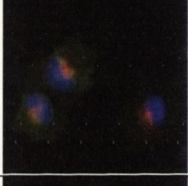
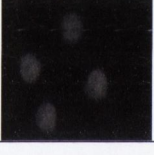
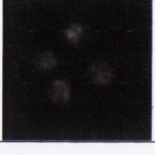
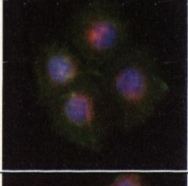
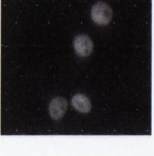
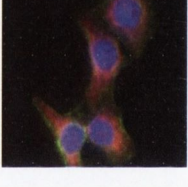
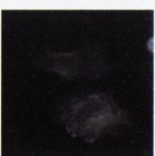
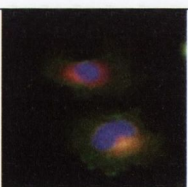
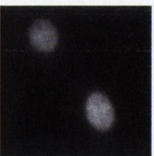
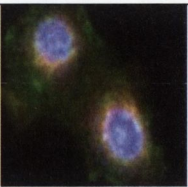
	Nucleus	F- Actin	Photosensitiser	Superimposed image
Untreated				
Temoporfin				
33				
34				
35				
36				
37				
43				

Figure 3.3.4.3 Examples of images for the uptake of glycoporphyrins in OE21 cells after 24 h, collected with the InCell imaging system. The images show the Hoechst-stained nucleus, phalloidin-stained F-actin and photosensitisers.

As previously mentioned, only six out of the eleven porphyrins evaluated showed any intracellular fluorescence and this was confirmed by the images collected with an InCell 1000 system (Fig 3.3.4.3). Temoporfin showed the strongest intracellular fluorescence intensity after 4 h of incubation and the intensity increased exponentially up to 24 h. The glycoporphyrins exhibited a lower intracellular intensity at all time points (Fig. 3.3.4.4), which clearly indicates that the uptake of these compounds by the cells is much slower. Nevertheless, a structure-uptake comparison of the compounds tested reveals some interesting differences. Three different types of glycoporphyrins were investigated: 5,10-disubstituted, 5,15-disubstituted and 5,10,15-trisubstituted. Of these, a larger number of the 5,10-disubstituted compounds showed uptake by the cells. The results suggest a relationship between the structure of the glycoporphyrins and cellular uptake, an important structural design criterion for the development of new photosensitisers. More extensive biological tests with these and related compounds and other cell lines must be performed. For the OE21 cells, the absence of phototoxic effects indicates the potential of the 5,10-disubstituted glycoporphyrins as possible imaging dyes that could be used under normal daylight conditions without adverse effects.

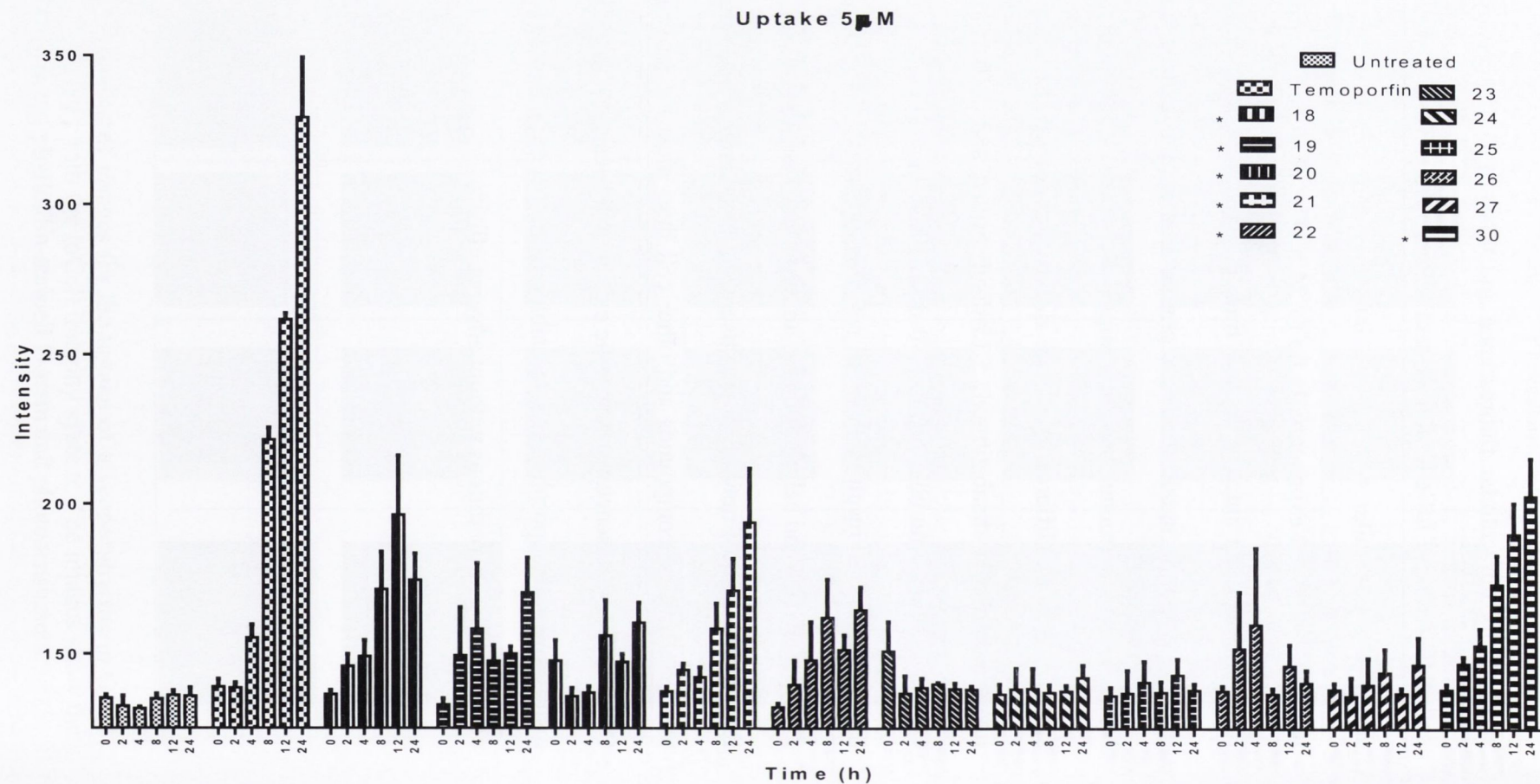


Figure 3.3.4.4 Graphical representation of the results obtained from InCell analysis for photosensitiser intensity in OE21 cells. Intracellular intensity of all the photosensitisers was followed over various incubation times. Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM. * represents the porphyrins were taken up.

After observing the lack of PDT toxicity of the glycoporphyrins, we were interested in investigating the relative singlet-oxygen production compared to our positive control, Temoporfin. Certain cell-morphology changes observed following irradiation led us to believe that some singlet-oxygen production was occurring, but in insufficient concentrations to induce cell death under these conditions. So we measured for relative $^1\text{O}_2$ production against Temoporfin of compound **36** (5,10 di- β -lactosamine-porphyrinato zinc(II)), which showed high uptake in the high content assay. This was measured by evolution of UV-vis absorption (read at 410 nm) of an aerated 1,3-diphenylisobenzofuran (DPBF) solution in the presence of the potential photosensitiser and Temoporfin illuminated by a homemade white light source set to an output of 415 W/m^2 [194, 229-231]. The glycoporphyrin was found to produce singlet oxygen but at a lower level than Temoporfin [194]. These results were consistent with the results of the toxicity studies and will be used to help optimize irradiation times and concentrations used for any further biological studies.

3.3.5 Library of novel phosphorous porphyrins

Porphyrins are ideal molecules for use as photosensitisers [232], however, one of the major issues affecting the general use of these molecules in the clinic is solubility of the compound in aqueous mediums. It has been reported that phosphorous porphyrins have the additional advantages of being able to penetrate biological barriers as well as increased solubility in both organic and aqueous solvents [233].

In our chemical synthesis laboratory, a library of novel phosphorous porphyrins was generated, the compounds tested were **44** - dihydroxo[5,10,15,20-tetrakis(1-ethylpropyl)porphyrinato]phosphorus(V) hydroxide ; **45** -

dihydroxo[5,10,15,20-tetrakis(1-methylpropyl)porphyrinato]phosphorus(V)
 hydroxide; **46** - dihydroxo[5,10,15,20-tetrakis(2-
 methylpropyl)porphyrinato]phosphorus(V) hydroxide; **47** -
 dihydroxo[5,10,15,20-tetrakis(n-butyl)porphyrinato]phosphorus(V) hydroxide;
48 - dihydroxo[5,10,15,20-tetraphenylporphyrinato]phosphorus(V) hydroxide
 (Fig. 3.3.5.1). These compounds were tested using the newly established HCS
 protocol as a primary screen.

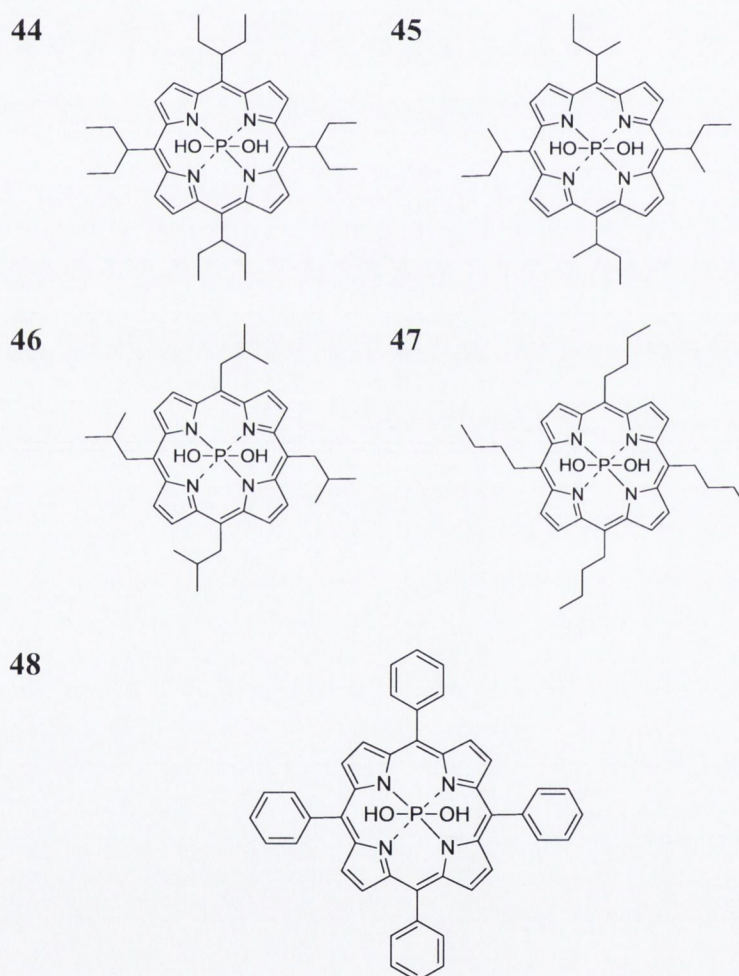


Figure 3.3.5.1 Chemical structure of the tested phosphorous porphyrins using HCS methodology.

Results showed that non-illuminated cultures showed no toxicity for all compounds, except **46** which showed significant dark toxicity (Fig. 3.3.5.1).

When cultures were illuminated we found that only compounds **46** and **48** showed activity in all cell lines (Fig. 3.3.5.2) shown by the reduction in nuclear area. No toxicity was observed upon treatment with vehicle alone (Fig. 3.3.5.2). These results were confirmed by the cell area analysis for all three analysed cell lines (Fig. 3.3.5.2).

Photosensitiser analysis showed that only compound **46** and **48** showed a significant intracellular integrated intensity in all cell lines (Fig. 3.3.5.3 A, C and E) and once more this parameter seems to indicate phototoxic efficacy. Intracellular area indicates that none of the phosphorus porphyrins tested occupy the same area as *m*THPC.

From the images collected in the InCell system we were able to visually confirm that only these two compounds were taken up by the cells (Fig. 3.3.5.4).

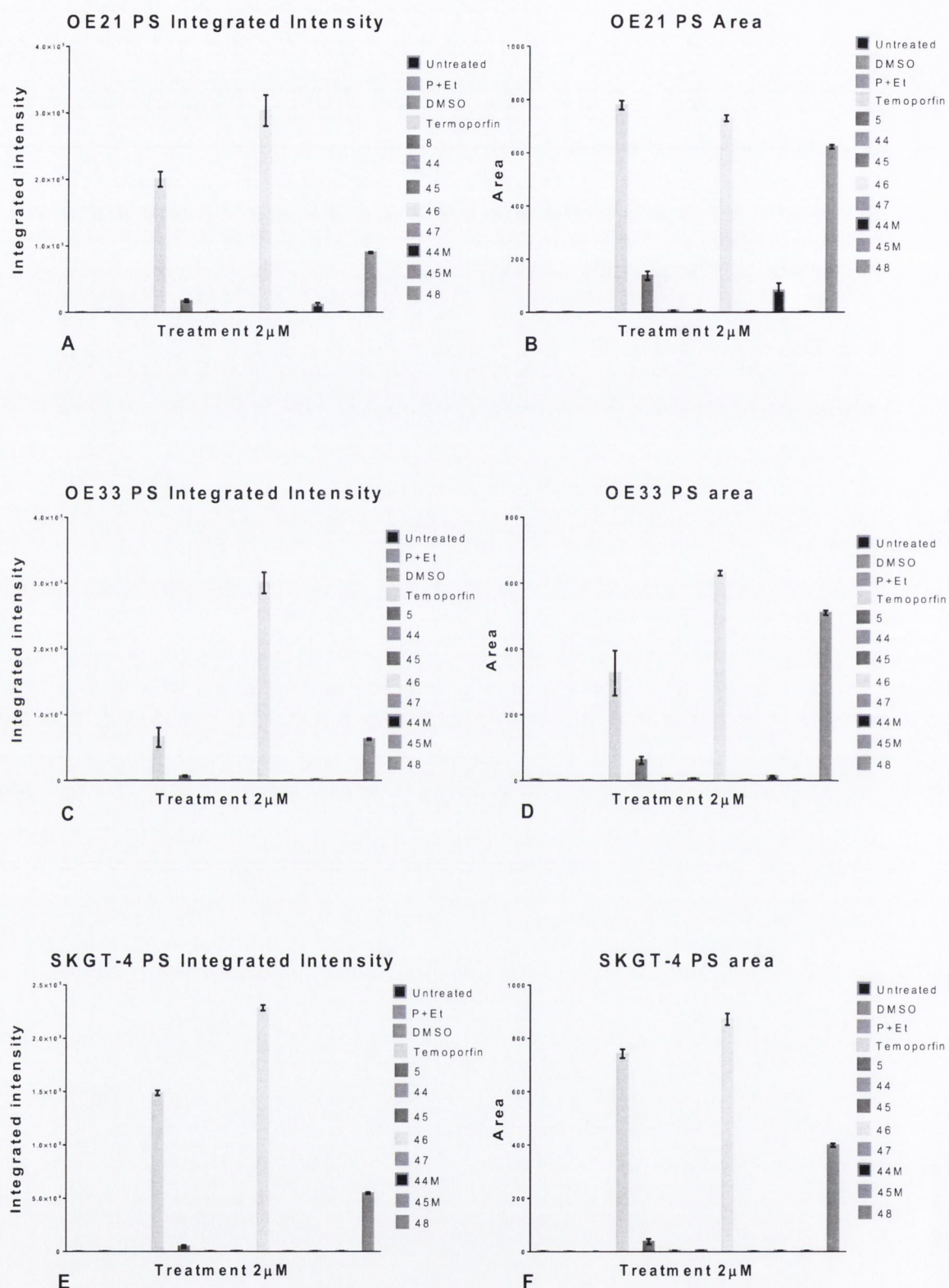


Figure 3.3.5.3 Results for porphyrin parameters in OE21 (A, B), OE33 (C, D) and SKGT-4 (E,F) cells treated with 2 μ M of Temoporfin and with equivalent concentrations of the test phosphoporphyrins. In terms of intracellular intensity (A, C and E) and area (B,E,F); Data are representative for three independent experiments and values are expressed in mean $\pm$ SEM.

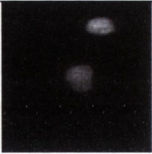
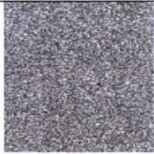
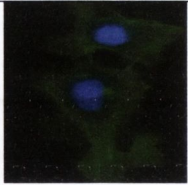
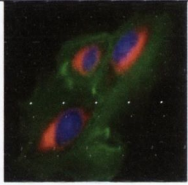
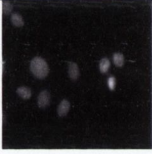
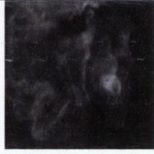
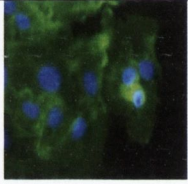
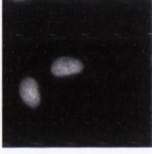
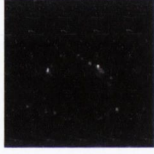
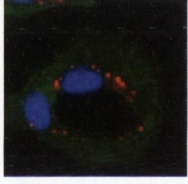
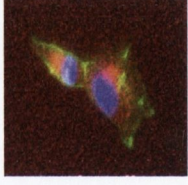
	Nucleus	F-actin	Photosensitiser	Superimposed image
Untreated				
Temoporfin				
44 45 47				
46				
48				

Figure 3.3.5.4 Examples of images for the uptake of phosphoporphyrins in SKGT-4 cells after 24 h of incubation, collected with the InCell imaging system. The images show the Hoechst-stained nucleus, phalloidin-stained F-actin and photosensitisers.

The identified hits (**46** and **48**) were analysed by MTS assay at a final post illumination time of 24 hours. From these results were able to determine that the various cell lines do, in fact, respond to photodynamic treatments differently both in illuminated and dark conditions (Fig. 3.3.5.5 and 3.3.5.6).

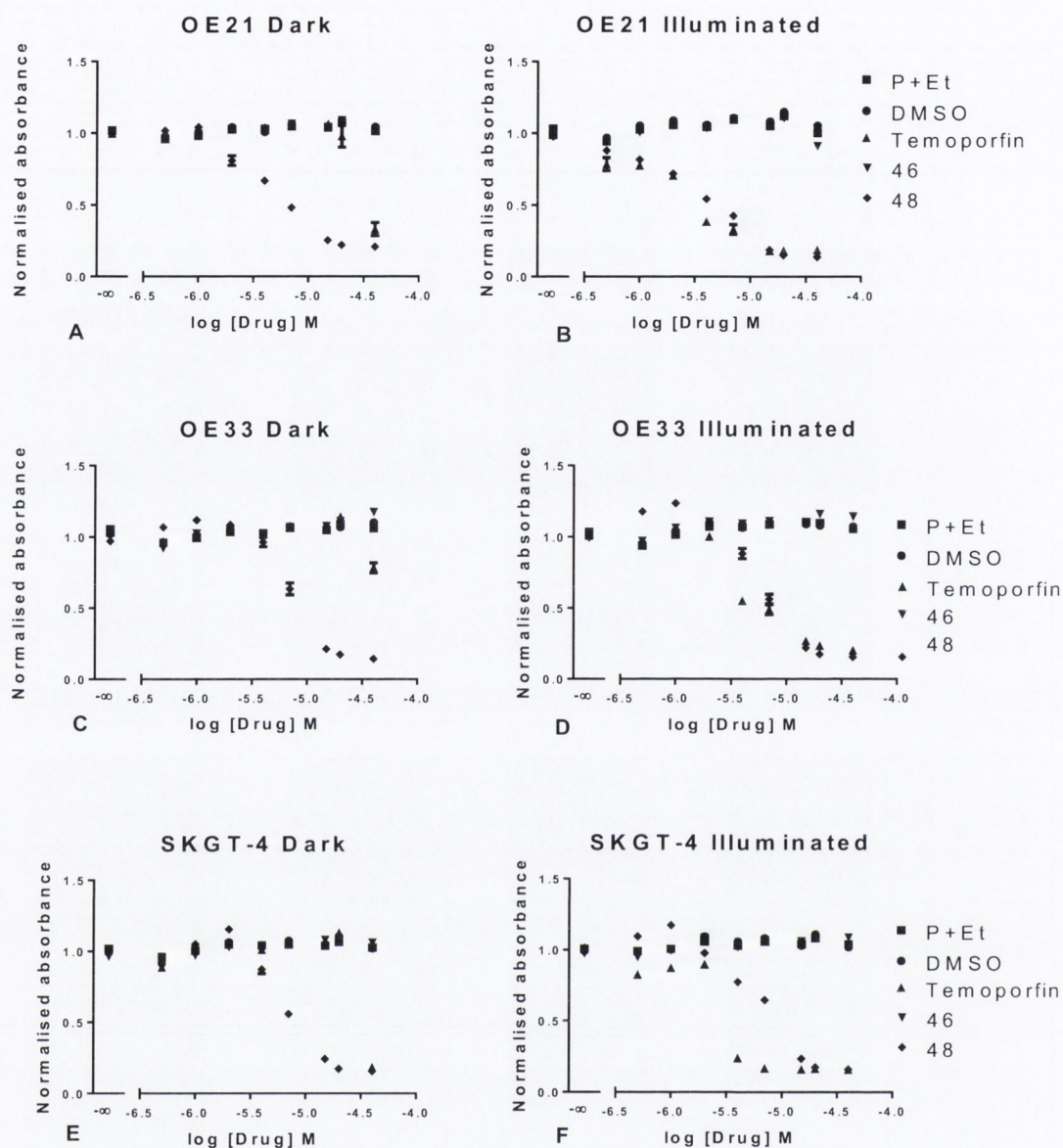


Figure 3.3.5.5 Graphical representation of the results obtained from the 24 hour MTS assay performed in the previously identified hits (**46** and **48**) as well as a non-phototoxic porphyrin previously tested (Screen 1 compound **5** - 5-(4-benzoic acid)-10,20-di(3-methoxyphenyl)porphyrin) tested in OE21 cells (A and B) OE33 cells (C and D) and SKGT-4 cells (E and F). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

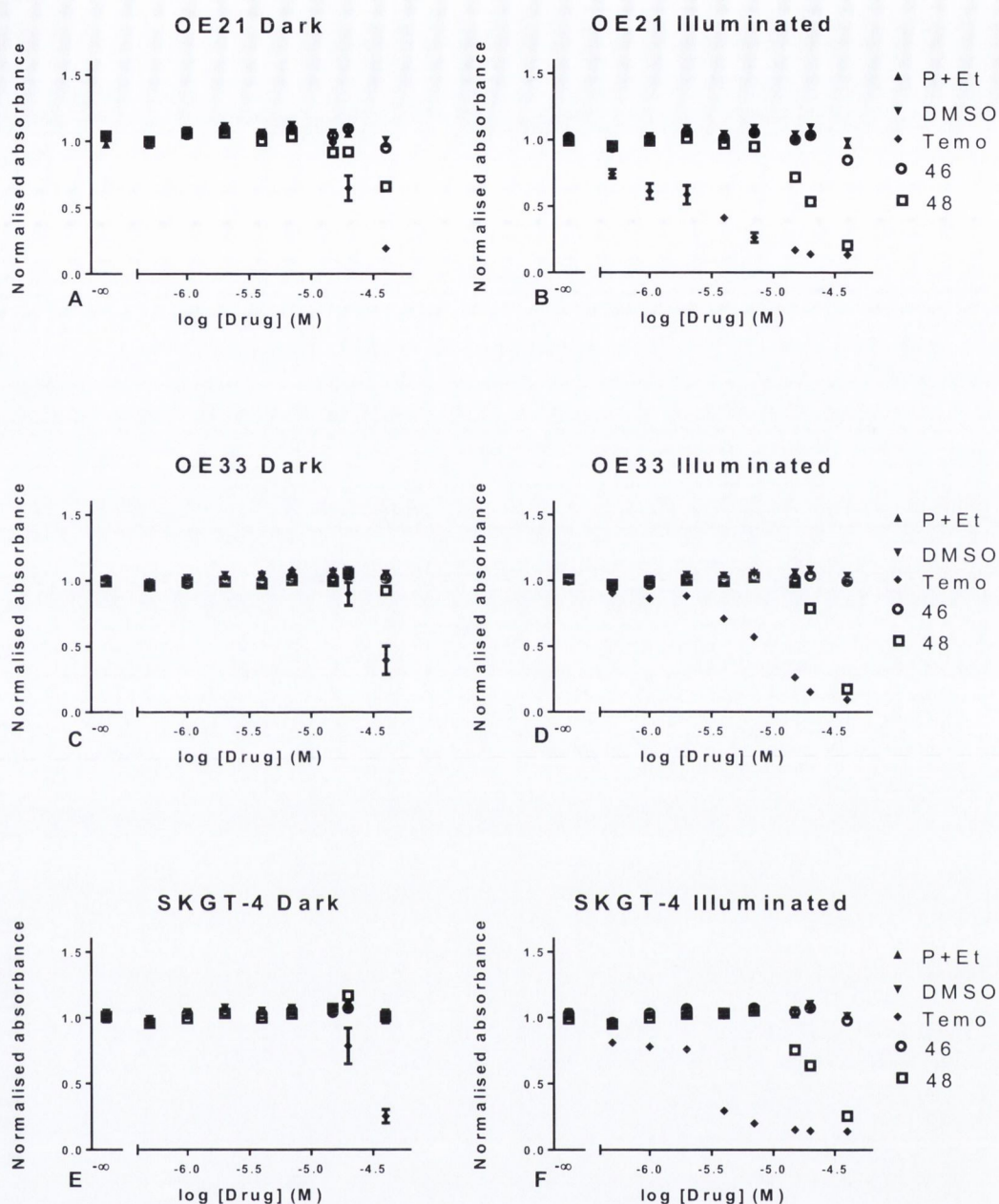


Figure 3.3.5.6 Graphical representation of the results obtained from the 24 hour MTS assay performed in the previously identified hits (**46** and **48**) as well as a non-phototoxic porphyrin previously tested (Screen 1 Compound **5** - 5-(4-benzoic acid)-10,20-di(3-methoxyphenyl)porphyrin) tested in OE21 cells (A and B) OE33 cells (C and D) and SKGT-4 cells (E and F). Data are representative of three independent experiments and values are expressed in mean ± SEM.

Table 3.3.5.1 Summary of dark and illuminated IC₅₀ values determined by MTS at 24 hours post illumination of all phosphoporphyrins that showed toxicity in the primary HCA screen. Values are representative of three independent experiments in OE33, OE21 and SKGT-4 cell lines.

OE21						
Dark						
	P+Et	DMSO	Temo	Sc1 5	46	48
IC ₅₀ (μM)	Ambiguous	3	22	2	5	Ambiguous
r ²	0.1883	0.1884	0.7853	0.209	0.9396	
Illuminated						
IC ₅₀ (μM)	Ambiguous	Ambiguous	2	Not converged	5	20
r ²	0.2985	0.3427	0.91		0.9692	0.9474
OE33						
Dark						
	P+Et	DMSO	Temo	Sc1 5	46	48
IC ₅₀ (μM)	3	12	Ambiguous	140	7	Not converged
r ²	0.1572	0.2272	0.5391	0.5108	0.9556	
Illuminated						
IC ₅₀	Ambiguous	Ambiguous	4	Ambiguous	6	22
r ²	0.2341	0.2584	0.9388	0.3695	0.9497	0.9372
SKGT-4						
Dark						
	P+Et	DMSO	Temo	Sc1 5	46	48
IC ₅₀ (μM)	Ambiguous	Ambiguous	21	2	7	8
r ²	0.2413	0.2968	0.917	0.5943	0.9583	0.1919
Illuminated						
IC ₅₀ (μM)	Ambiguous	Ambiguous	3	Ambiguous	7	21
r ²	0.3136	0.3431	0.9715	0.4834	0.9513	0.9121

The results further validate that OE21 and SKGT-4 cells are more susceptible to dark toxicity from *m*THPC. No such dark toxicity was seen for OE33 cells making them much more resistant to these types of compounds (Table 3.3.5.2). The results also confirm the strong dark toxicity of **46** making this compound too toxic for further development. Compound **48** on the other hand showed high dark toxicity

comparable to Temoporfin, however, it showed no significant decrease in illuminated IC_{50} hence it is also unsuitable for further development.

In addition, the results confirm the lack of toxicity of the negative control and the vehicles used; if an IC_{50} could be determined the r^2 values are too low in order for these to be deemed statistically valid (Table 3.3.5.1).

We tested several phosphorus porphyrins hoping they would offer significant advantages, however, these failed to materialise. They show increased dark toxicity in the case of **46** and they did not show any increase in solubility. These chemicals will be taken to the chemical synthesis lab in order pursue an alternate chemical synthetic route in order to generate new potential phosphorous photosensitisers.

From our efforts in identifying new potential PDT drugs we screened a total of 48 compounds against our oesophageal cancer cell models. From all the compounds tested only 10 (**2**, **4**, **8**, **14**, **15**, **22**, **24**, **27**, **46** and **48**) showed phototoxicity towards our oesophageal cell models (Table 3.3.1). From these we were able to determine that, when compared to *m*THCP, compound **4** had a marginal lower IC_{50} against OE33 and SKGT-4 cell lines; Compounds **8** and **15** also had a marginal lower IC_{50} against SKGT-4 cells; Compound **14** showed a lower IC_{50} in OE21 and OE33 cell lines and compound **27** showed a lower IC_{50} in SKGT-4 and OE33 cell lines. On the other hand compounds **22**, **24**, **46** and **48** showed a significantly higher IC_{50} against all cell lines when compared to the action of Temoporfin. As expected compound **2** showed the same phototoxic profile as Temoporfin.

3.4 Development of oesophageal 3D spheroids and assessment of their suitability for screening porphyrins

Most laboratory evaluations of pre-clinical drug toxicity are executed in cultures of cells in monolayers or, in the case of non-adherent cells, in cell suspensions. Yet, it is a known fact that the responses to toxicants of cell monolayers are radically different to those of cells in solid tumours [234]. These responses are due to the dramatically different architecture of cells altering many parameters such as nutrient and oxygen availability, gene expression and exposure to treatment or drug [234].

It is widely believed that solid tumours often have a necrotic and acidic core [235, 236] this leads to poor penetration and diffusion [237] of anticancer drugs into deep regions of tumours leading to poor treatment outcome and drug resistance [235, 238]. In fact, it has been estimated that 60% of human solid tumours have regions of hypoxia and/or anoxia [236, 239]. These hypoxic/anoxic states have been linked to poor prognosis and low patient survival rate after anticancer therapy [239].

In the context of PDT these hypoxic regions adversely affect the effectiveness of the treatment [235, 240, 241], in fact in the absence of oxygen PDT will have no therapeutic effect [125, 242, 243].

Cell monolayers grown in cell culture flasks are cultured in optimum conditions of not only temperature but also O₂ availability [244, 245] and therefore these monolayers may offer limited information regarding the effect of PDT in solid tumours. For this reason it is necessary to explore other disease models that are more comparable to solid tumours in the body; an example of these is the use of

spheroids for the evaluation of PDT [103, 235, 239]. Spheroids have the potential to be the missing link for the evaluation of potential anti-cancer compounds between cells growing in monolayers and tests in animal models [235, 239, 246].

Though there are many examples of spheroid cultures for a myriad of different cancers in the literature [247-251] very few focus on oesophageal cancer [252]. In addition, most of the techniques used in the formation of tumour mimics are cumbersome and difficult to master; hence, they may be often unsuitable as an integrate part of a drug screen.

With our interest in oesophageal cancer and drug screening we tested the suitability of the new technology, Happy cell® (Biocroi, Ireland), in order to cultivate oesophageal cancer spheroids *in vitro*, and test the effect of various photosensitisers in these 3D models of oesophageal cancer.

Happy cell® is a novel liquid scaffold that allows for cells to form spheroids *in vitro* [253]. It is much like a liquid medium routinely used cultures but stimulates cells to aggregate into 3D tumour structures.

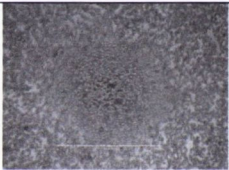
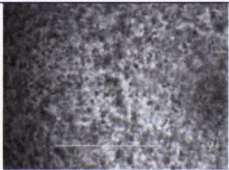
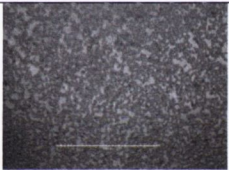
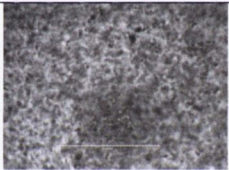
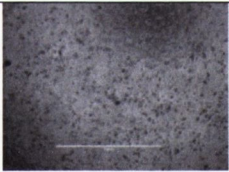
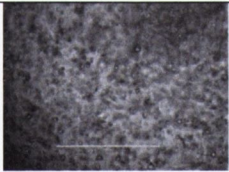
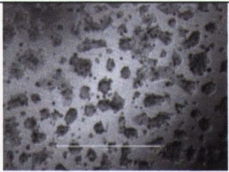
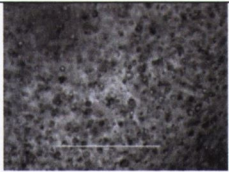
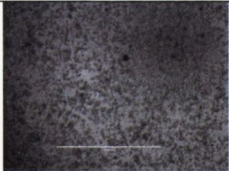
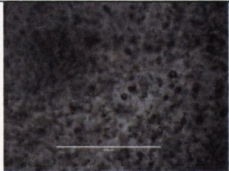
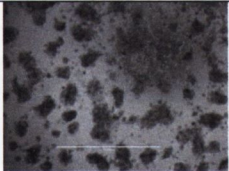
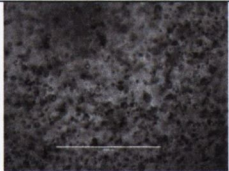
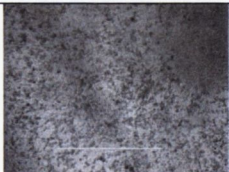
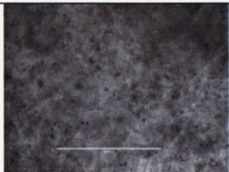
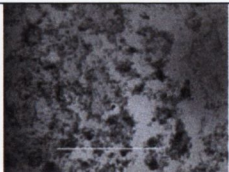
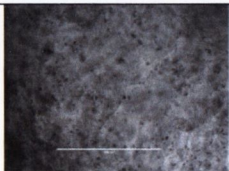
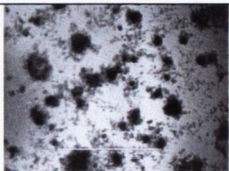
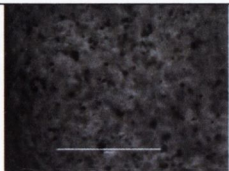
Different concentrations of OE21 and OE33 cells (1.5×10^4 , 3×10^4 , 7.5×10^4 and 15×10^4 cells per well) were seeded into 96 well low adherence plates (Sarstedt, Germany) and Nunclon plates and left for up to 7 days, assessment of spheroid formation was done by visual inspection. We were able to visually confirm that a concentration of 7.5×10^4 cells per well would yield spheroids within 48 hours (Table 3.4.1), another 24 hours was added to the experiment in order to let the spheroids mature.

Results showed that in low attachment plates, OE21 cells grew in monolayer when cultured in RPMI (data not shown) while OE33 cells form hollow

“mounds” of cells (Fig. 3.4.1). Both cell lines cultured in low attachment plates and Happy cell® grown in tight truly 3D spheroids; Validated both by wide field (Table 3.4.1) and confocal microscopy (Fig. 3.4.1).

We also tested SKGT-4 cells yet these cells failed to form any significant 3D structures using this method (data not shown). These results confirm that Happy Cell® is a simple method of generating spheroids using cells that have a natural propensity to aggregate in 3D structures and does not artificially force cells into aggregates.

Table 3.4.1 Results obtained from 7.5×10^4 cells per well of OE33 cells seeded under various conditions and monitored for 4 days.

	Nunc Plate RPMI	Nunc plate Happy cell®	Low attachment RPMI	Low attachment Happy cell®
Day 0				
Day 1				
Day 2				
Day 3				
Day 4				

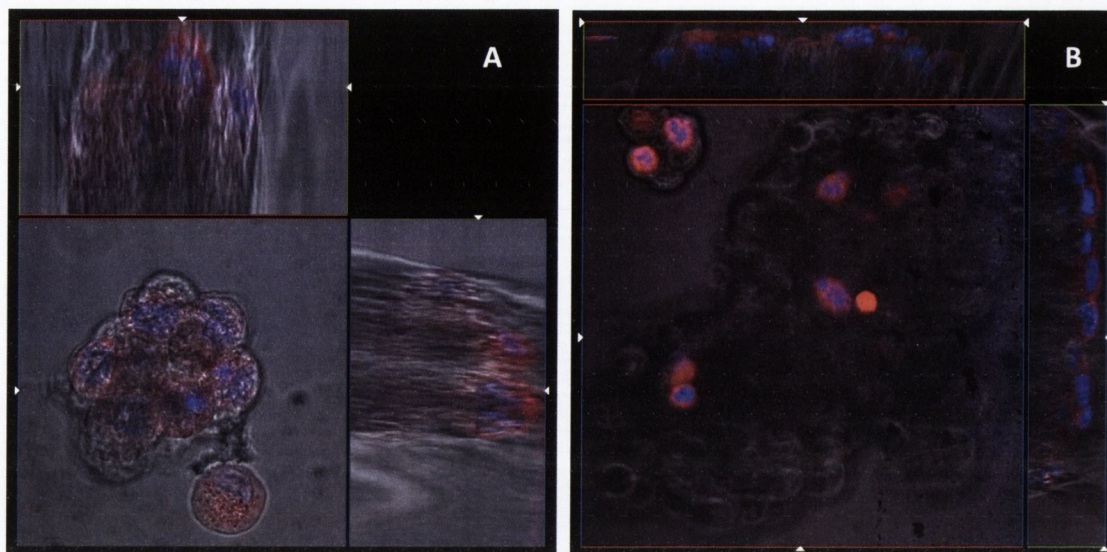


Figure 3.4.1 Confocal Images collected for OE33 spheroids cultured in Happy Cell (A) and RPMI (B) in low attachment plates. The cultures were stained with 12 μ M Temoporfin (red cytoplasm) and Hoescht (blue nucleus).

When cells were treated with 12 μ M Temoporfin for 24 h we were able to determine that this incubation time is sufficient time for the phosphosensitiser to penetrate to the core of the spheroids (Fig. 3.4.2).

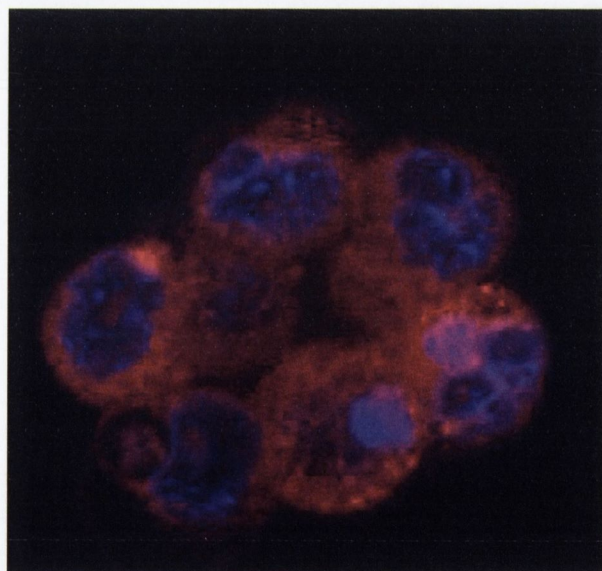


Figure 3.4.2 Confocal image collected for OE33 spheroids grown in Happy Cell in low attachment plates. The cultures were incubated with 12 μ M Temoporfin (red cytoplasm) and Hoescht (blue nucleus).

The need for a protocol for inactivating Happy cell® was also addressed. This step was essential as the removal of treatment medium before illumination is necessary to ensure the PDT effect was due to PS that had been taken up by the cells during incubation and not due to PS present in the outside of the cells. This was achieved using the supplied Inactivation Solution (Biocroi, Ireland).

Cells were seeded as described in materials and methods, at this point 10µL of inactivation solution (at different concentrations) and 20µL of MTS were added per 100µL of Happy cell® and incubated for up to 4 hours. The plate was then read at various time points. It was determined that a concentration of 0.625g/L of inactivation solution was sufficient to sediment the spheroids from solution and would not significantly affecting the viability of the spheroids (Fig. 3.4.3). The toxicity observed (at higher concentrations) may have been a direct effect of the fact that the inactivation solution being supplied in an acidic mixture.

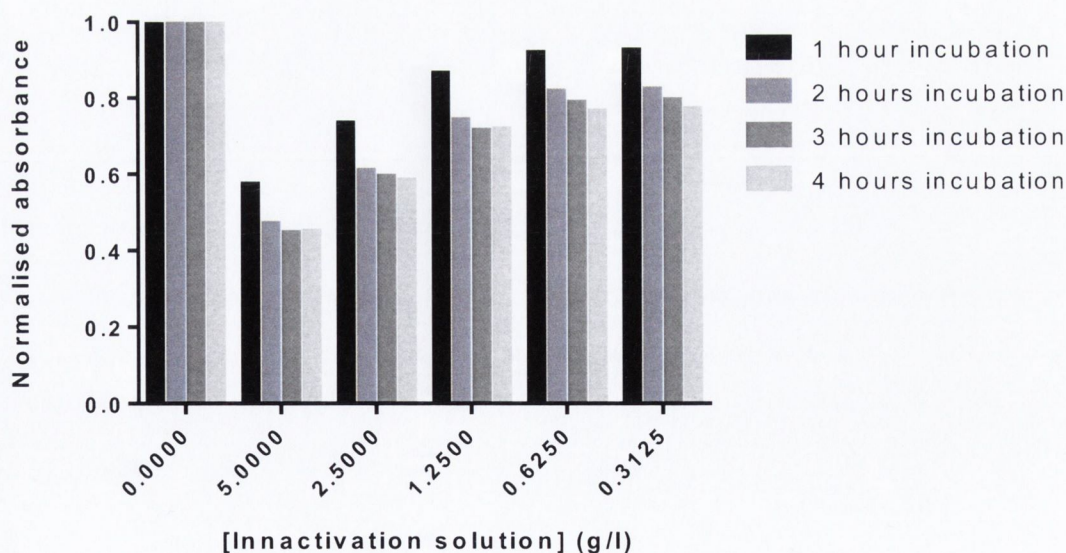


Figure 3.4.3 Graphical representation of the viability of OE33 spheroids incubated with different concentrations of inactivation solution for various time points.

With protocols established for generating and manipulating oesophageal spheroids we proceeded to test the PDT efficacy of different photosensitisers.

As a first step, monolayer and spheroid cultures of OE21 and OE33 were treated with 12 μ M a positive control (Temoporfin), a negative control (screen 2 compound **5** - 5-(4-benzoic acid)-10,20-di(3-methoxyphenyl)porphyrin) known be taken up by cells but showed no phototoxicity) [254], and two new compounds, hits from our previous porphyrin screen (**46** - Dihydroxo[5,10,15,20-tetra(2-methylpropyl)porphyrinato]phosphorus(V) hydroxide and **48** - dihydroxo[5,10,15,20-tetraphenylporphyrinato]phosphorus(V) hydroxide) as well as equivalent amounts of vehicles (mixture of propylene glycol (40%) and ethanol (60%) used to dissolve Temoporfin and DMSO used to dissolve all other test compounds).

These compounds were tested by MTS 24 hours after illumination in spheroids and monolayer cultures of OE33 and OE21 cells d (Fig. 3.4.4). Results showed that when in 3D both OE21 and OE33 are much more resistant to the action of all compounds tested (Fig. 3.4.4 C and D); none of the vehicles used or the negative control exhibited toxicity in any of the cell culture types tested. Reflecting the behaviour of the cells in monolayer OE21 cells grown in spheroid form were more sensitive to the action of photosensitisers.

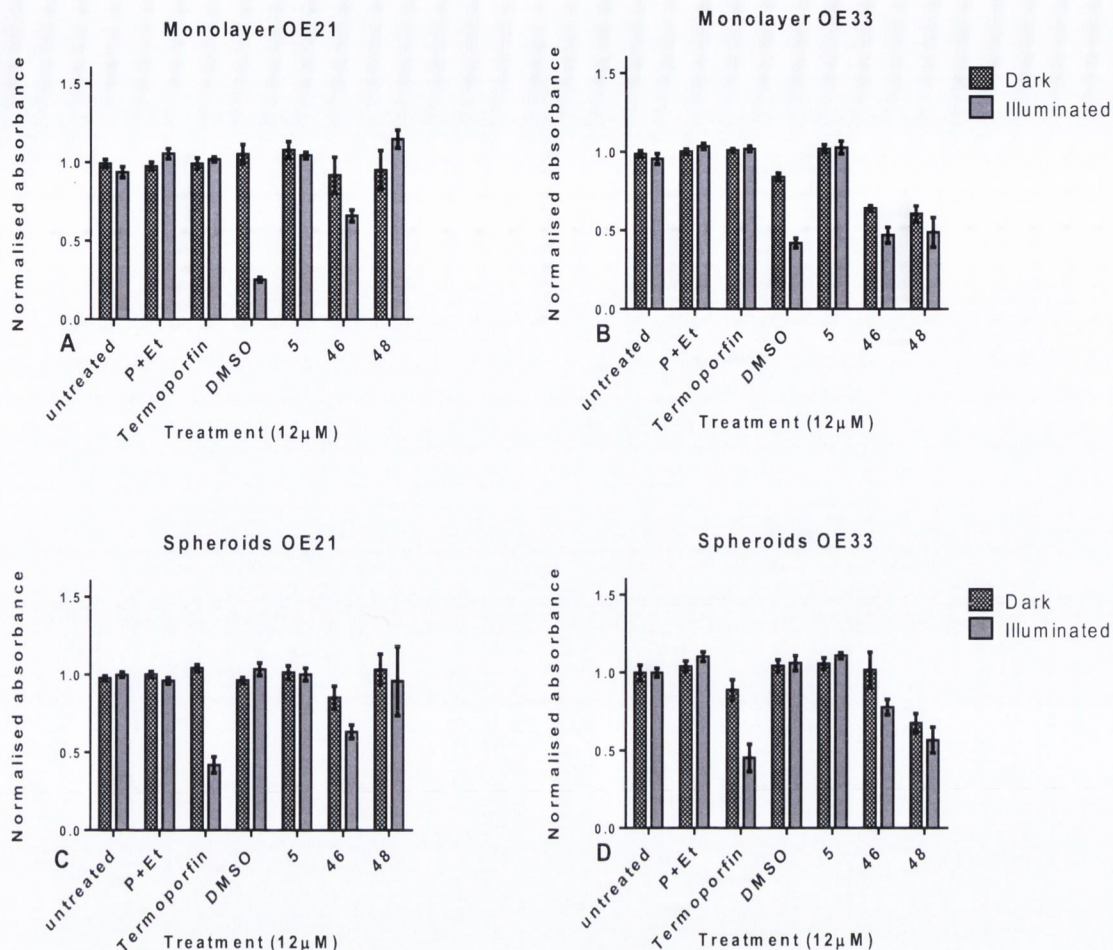


Figure 3.4.4 Graphical representation of the results obtained for the test of photosensitisers, negative control and vehicles by MTT 24 hours after illumination. Cultures of OE21 (A and C) and OE33 (B and D) were tested as monolayers (A and B) and spheroids (C and D). Data are representative of two independent experiments and values are expressed in mean $\pm$ SEM.

Having been able to establish that the experimental protocol showed efficacy of potential PS we set out to determine the IC_{50} of the tested drugs using MTS assay for 3D models of oesophageal cancer and compare these with IC_{50} determined for adherent cultures. The compounds mentioned above were used to determine IC_{50} of treated OE33 spheroids.

As expected the results showed that when treating spheroids with various concentrations of photosensitisers there is a significant increase in the IC₅₀ when compared to the same cells grown in monolayer (Fig. 3.4.5 and Table 3.4.2).

Temoporfin showed an increase from 4 μ M in monolayers to 17 μ M in spheroid cultures of OE33 cells (Fig. 3.4.5 and Table 3.4.2). All other compounds showed ambiguous results as the range of concentrations used was not wide enough to determine a statistically significant IC₅₀.

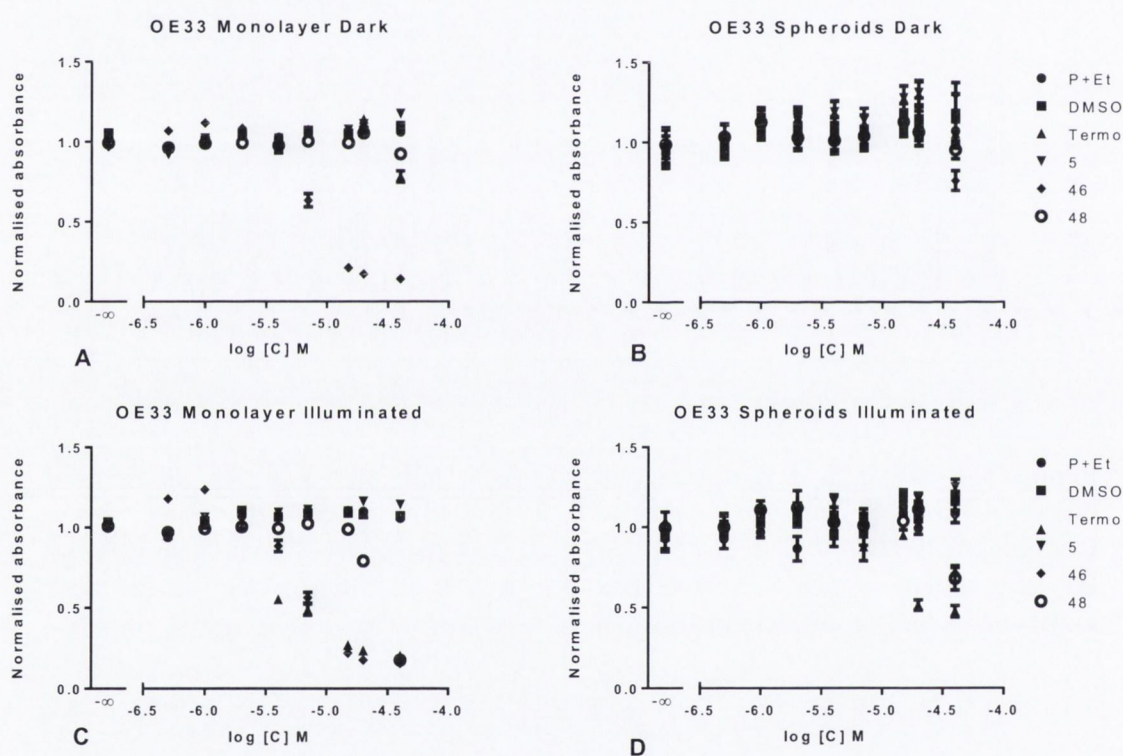


Figure 3.4.5 Graphical representation of the results obtained from the 24 hour MTS assay performed in the previously tested compounds in OE33 cells in monolayers (A and C) and in spheroids (B and D). Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

Table 3.4.2 Summary of the results obtained for IC₅₀ values in monolayers and in spheroid cultures of OE33 cells. Results are representative of three independent experiments.

Compound	Monolayer		Spheroids	
	Dark	Illuminated	Dark	Illuminated
Temoporfin	Ambiguous	4 µM	Ambiguous	17 µM
46	7 µM	6 µM	Ambiguous	Ambiguous
48	Not converged	22 µM	Interrupted	Ambiguous

These results are in accordance with results reported in the literature and imply a significant drug resistance in laboratory grown 3D structures [255-258]. However, a wider range of concentrations should be tested in the future to account for this increased resistance to PDT and to increase the reliability of IC₅₀ value.

In order to further investigate structural characteristics of the spheroids it was necessary to fix these for long term storage, analysis and further staining. Hence a reliable fixation protocol was needed in order to preserve these spheroids. It is known that a long incubation in PFA solution is needed to acceptably preserve 3D structures. We tested two concentrations of PFA, 4% routinely used in our laboratory to fix cell monolayers, and 2% over a 24 hour period. We found that if a 4% concentration of PFA was used the formaldehyde would precipitate from solution and would significantly affect imaging of the spheroids (Fig. 3.4.6 A), while a lower concentration would still be suitable for fixation with but would not yield precipitates (Fig. 3.4.6 B).

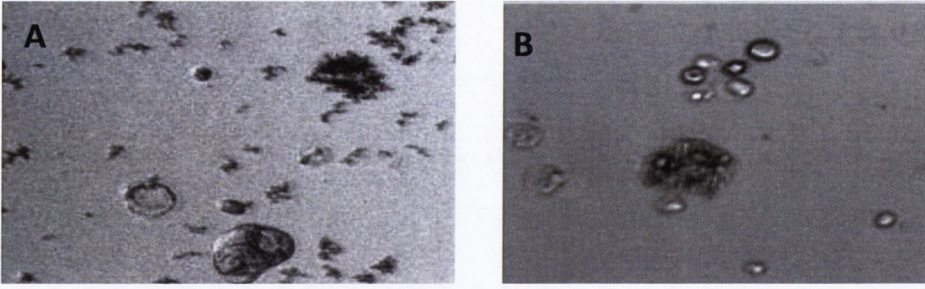


Figure 3.4.6 Brightfield images obtained for spheroid cultures of OE33 cells fixed in 4% PFA (A) and 2% PFA (B). Spheroids were fixed for 24 hours prior to evaluation.

In order to stain F-actin on the cells that were in spheroids with phalloidin yet another protocol had to be optimized. Fixed spheroids were stained with phalloidin for 24 hours, at this point staining solution was removed and spheroids were washed once in PBS. The spheroids were resuspended in PBS and were then ready for imaging or long time storage.

Our results show that this protocol is sufficient to stain our 3D structures with our desired fluorophores (Fig. 3.4.7).

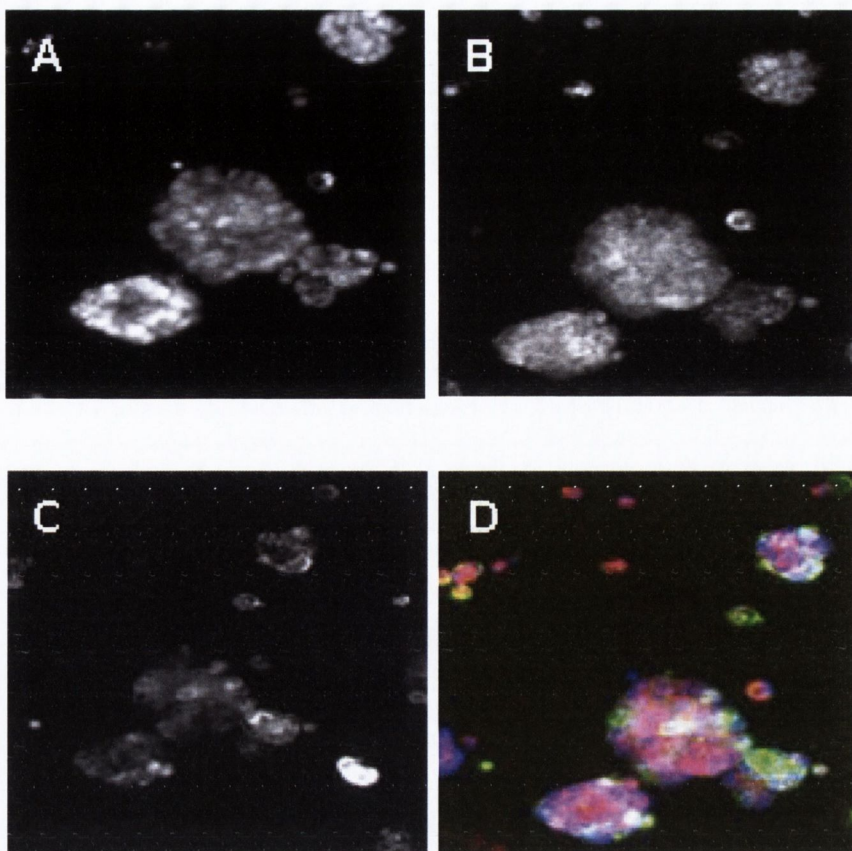


Figure 3.4.7 Images collected from the InCell 1000 system for OE33 spheroids incubated with 12 μ L Temoporfin (B) fixed in 2% PFA and further stained with Hoescht (A) and Phalloidin (C) and superimposed image (D).

We were able to establish a robust and consistent protocol for the generation and handling of spheroids for oesophageal cells. We tested photosensitisers in these structures and were able to confirm that not only do they follow the behaviours of the respective monolayers but also showed a significant increased resistance to PDT agents.

Spheroids were fixed using 2% PFA overnight and, following the removal of the fixative, further stained (overnight) with phalloidin and Hoescht for analysis in fluorescent imaging systems.

3.5 Assessment of a library of compounds as possible cellular fluorophores

In past decades, molecular imaging technology has become a central tool in biology, particularly in bioassays, cell imaging and cancer research [134, 135, 259-261]. The development of novel luminescent probes for cellular and tissue analysis, that are efficient and kinetically stable, is vital to the advance of biological and clinical research. Rare earth metal complexes are now versatile targets in many areas of research, such as photochemistry, biomedicine and nanotechnology [262-269].

HCS [270], as previously discussed, is based on fluorescent materials targeting cellular targets, and is widely used in biomedicine, cancer research and drug development. These automated systems provide quick analyses and generate a massive amount of information in a relatively short period of time. For example, they can deliver 10^8 individual physiological measurements at the cellular level under days [271]. This integrated approach has led us to develop and apply an HCS protocol for the development of PDT agents and has been used to image oesophageal spheroids in an attempt to develop ever more reliable PDT drugs.

Small fluorescent molecules offer significant potential in the area of fluorescent imaging and analysis [272] and can be easily synthesized on a large scale making them commercially viable and cheap [273].

With our interest in cellular imaging we tested a library of 10 small molecules as possible fluorescent stains to be applied to our primary HCS screen and spheroid structures. **1** - 1,10-phenanthroline-5,6-dione ; **2** - 2-(4-bromophenyl)imidazo[4,5-f]-1,10-phenanthroline ; **3** - 2-{4-[1-(2-

trimethylsilyl)ethynyl]phenyl)imidazo[4,5-f]-1,10-phenanthroline ; **4** - 2-(4-pyridinyl)imidazo[4,5-f]-1,10-phenanthroline ; **5** - 2-(4-bromophenyl)imidazo[4,5-f]-1,10-phenanthroline europium(III) complex ; **6** - 2-{4-[1-(2-trimethylsilyl)ethynyl]phenyl)imidazo[4,5-f]-1,10-phenanthroline europium(III) complex ; **7** - 2-(4-pyridinyl)imidazo[4,5-f]-1,10-phenanthroline europium(III) complex ; **8** - (2-(4-bromophenyl)-N-hexyl)imidazo[4,5-f]-1,10-phenanthroline zinc(II) complex ; **9** - 2-(4-bromophenyl)imidazo[4,5-f]-1,10-phenanthroline zinc(II) complex ; **10** - 2-(4-pyridinyl)imidazo[4,5-f]-1,10-phenanthroline zinc(II) complex. Of these, compounds **1** to **4** are simple phenanthroline small molecules; **4** to **7** are phenanthroline europium(III) complexes and **8** to **10** are phenanthroline zinc(II) complexes (Fig. 3.5.1).

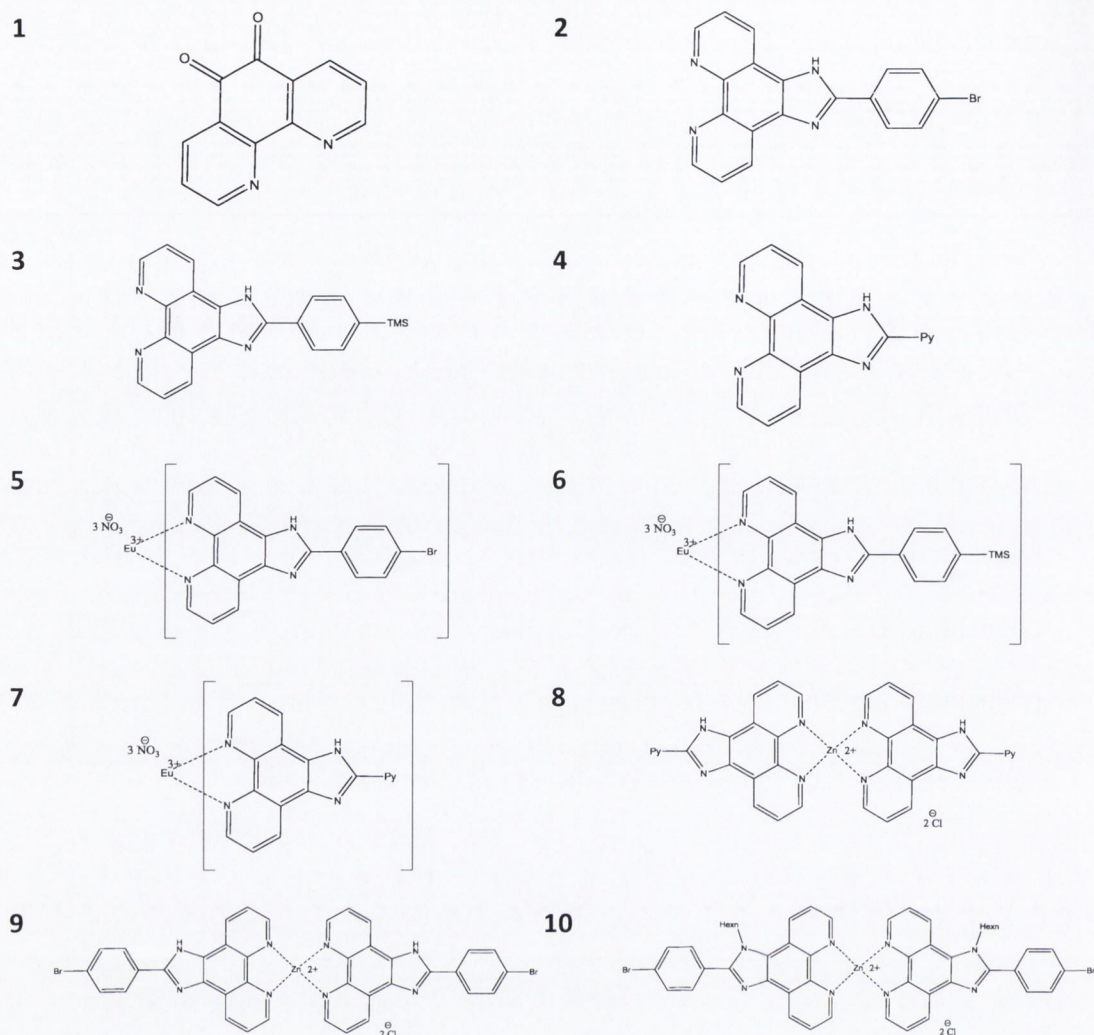


Figure 3.5.1 Chemical structures of all the small molecules tested as possible fluorescent stains.

3.5.1 Phenanthroline-based small molecules

Phenanthroline molecules are natural chelating agents commonly used as building blocks for many biologically relevant molecules due to their strong affinity for transition metal ions [274-277]; these molecules are also excellent protease inhibitors [278-280]. Though they display low fluorescent in their native state, when conjugated with other chemical species, they switch to a high

tuneable fluorescence [132, 254, 281] making these species excellent candidates to be used in HCS and, subsequently, integration into an HCA PS discovery tool.

We found that small molecules such as phenanthroline derivatives can be used as versatile materials for cellular imaging and cancer screening studies. They show strong absorption in the 280–400 nm region, exhibit high fluorescence upon excitation and large Stokes shifts. These optical properties do not alter drastically with changes of the solvent systems and the compounds are stable in aqueous media with no decay in fluorescence over time [273]. From our studies we found that compounds **2** and **3** are soluble in aqueous phosphate buffered saline solution (PBS) and exhibit a strong fluorescence in PBS at pH 7.4 upon excitation at 405 nm (Fig. 3.5.1.1).

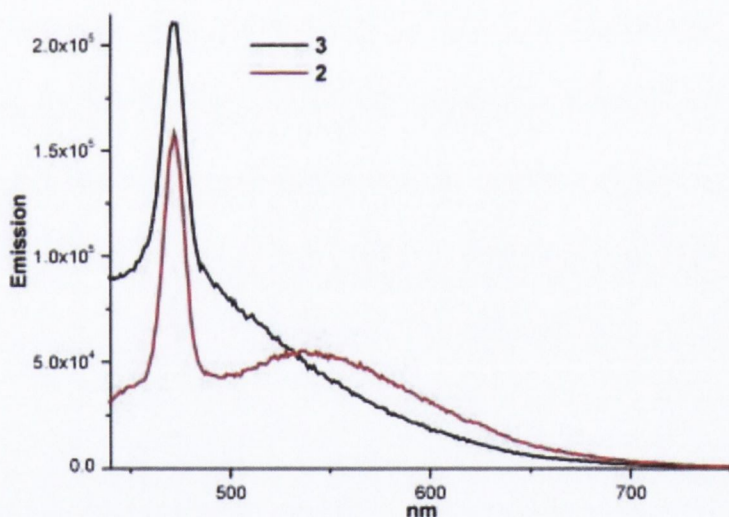


Figure 3.5.1.1 Emission spectra of **2** and **3** at excitation of 405 nm in PBS at pH 7.4 (10 μ M).

Studies on localization, incubation times, concentration ranges, and toxicity were carried out in two cell lines OE21 and HET-A [273]. An analysis and evaluation of live HET-1A and OE21 cells stained with the novel compounds

at different time points (5 minutes to 24 hours) and concentration ranges (2–27 μM) was performed with HCS. Remarkably, cellular images could be collected after 5 min of incubation for both compounds in contrast to some metal complexes [282] for which longer times are required (Fig. 3.5.1.2).

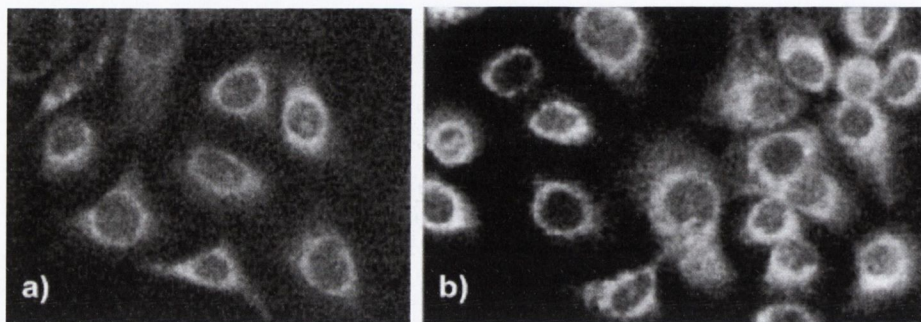


Figure 3.5.1.2 InCell images of the OE21 live-cells at 405 nm irradiation and 535 nm emission: (a) **2**, 5 min, 9 μM ; (b) **3**, 20 min, 27 μM .

Dyes **2** and **3** appeared to accumulate in the ER (a vital cellular organelle [283] which is involved in the regulation of crucial cell events such as intracellular signalling having a calcium storage function involving release and uptake of Ca^{2+}) and participates in the initiation of apoptotic cell death pathways [284, 285].

The intracellular localization of the dyes **2** and **3** was studied by confocal microscopy. Live HET-1A cells were incubated with ER tracker red and stained with **3** (18 μM). Dye **3** and ER tracker did not show spectral overlap, and the superimposed images of the red ER stain and **3** provided further indication that the latter accumulates in the endoplasmic reticulum (Fig. 3.5.1.3).

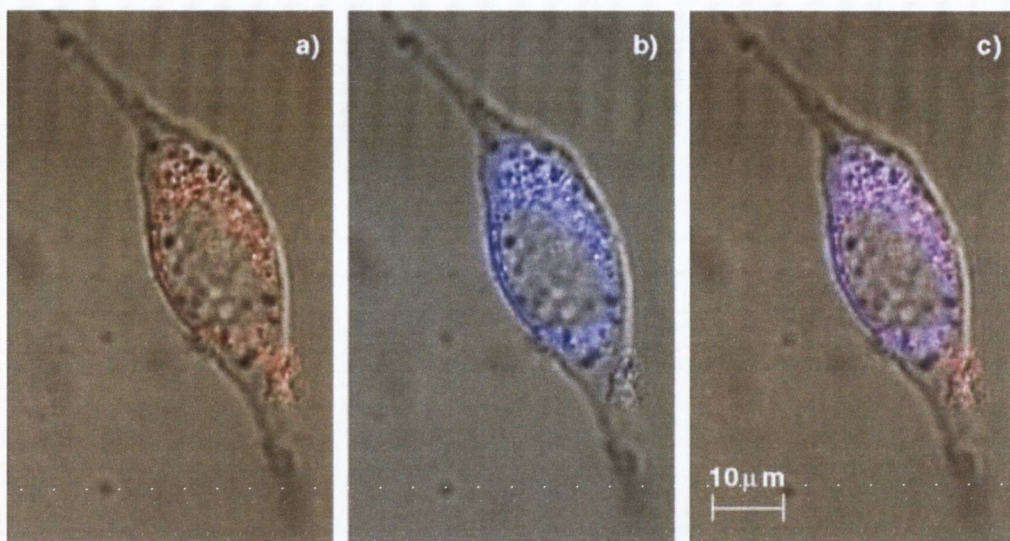


Figure 3.5.1.3 Confocal fluorescence images of HET-1A live-cells incubated with: (a) **3**; (b) red ER tracker; (c) superimposition of **3** and red ER tracker.

Similar results were obtained for **2** (Fig. 3.5.1.4 and 3.5.1.5). The experiments were carried out in OE21 live cells and showed the same localization in both cell lines. Furthermore, we carried out the same *in vitro* experiments using fixed cells with the same outcome. Noteworthy, ER-tracker has to be administered to living cells, incubated, and only then can cells be fixed if the experiment requires it. In our case the compounds **2** and **3** can be used to stain living as well as fixed cells, both appear to occupy the same intracellular localization.

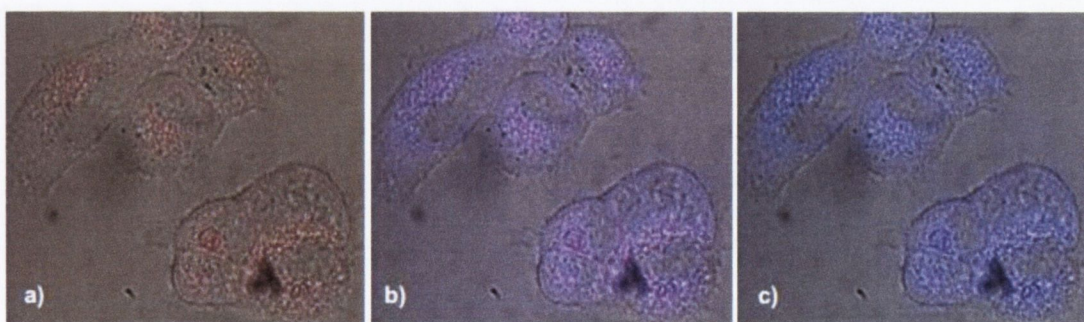


Figure 3.5.1.4 Confocal images for live OE21 cells incubated with compound **2**; a) ER-tracker red; b) Superimposition of **2** and ER tracker red; c) compound **2**

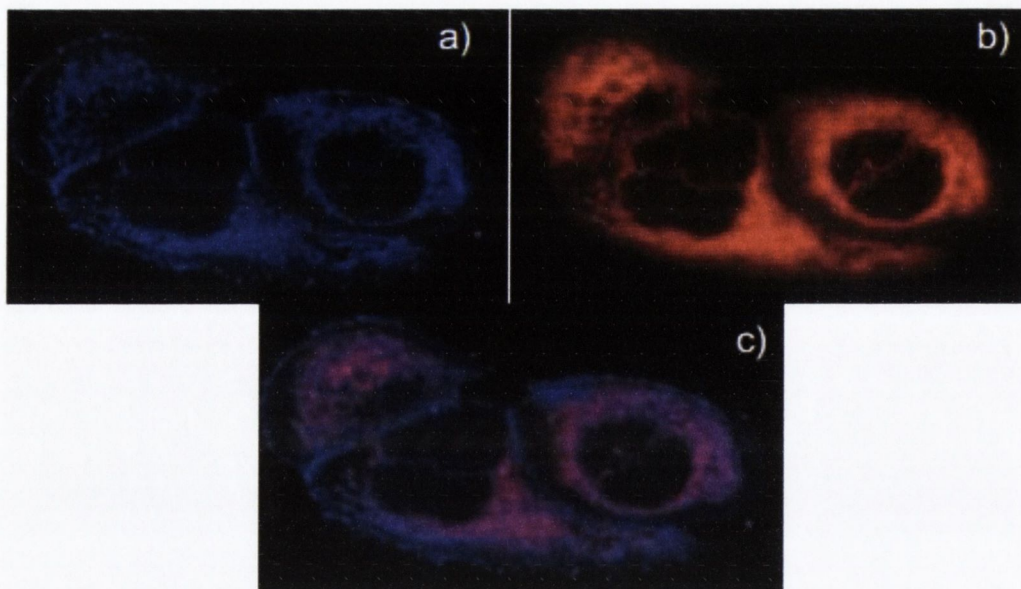


Figure 3.5.1.5 Confocal fluorescence images of fixed OE21 cells incubated with: a) compound **2**; b) ER-tracker red; c) Superimposition of **2** and ER tracker red.

Additionally, both dyes show remarkable cytotoxicity in cancer cells and may possibly be used as potential therapeutics for cancer treatment (Fig. 3.5.1.6 A and B). Cytotoxicity tests were performed on both cell lines and carried out using an MTT assay (Fig 3.5.1.6) and vehicle controls were implemented (Fig. 3.5.1.6 C) the concentrations tested showed no significant toxicity in any of the cell lines tested (Figure 3.5.1.6 C). The cells were incubated with **2** and **3** for 24 h at a concentration range of 0.5–15 μM in DMSO (Table 3.5.1.1).

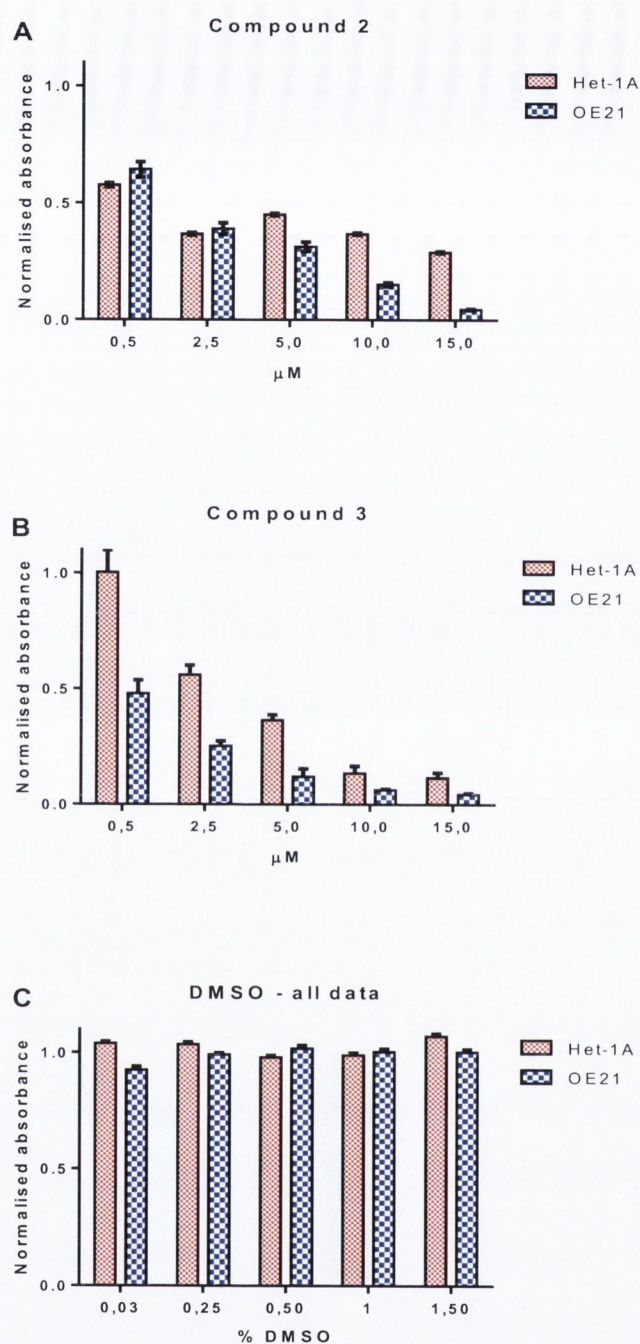


Figure 3.5.1.6 Graphic representation the toxicity results by MTT test for compound **2**, **3** and vehicle used. Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

Table 3.5.1.1 Cytotoxicity of **2** and **3**. IC₅₀ values for OE21 and HET-1A cell lines.

Cell line	Compound	IC ₅₀ (μM) ± SE
OE21	2	0.77 (0.29) ± 0.13
Het-1a	2	3.15 (1.18) ± 0.08
OE21	3	0.44 (0.16) ± 0.005
Het-1a	3	3.02 (1.11) ± 0.32

Overall, the cytotoxicity shown is very low and comparable to established anti-cancer drugs, for example, cisplatin, carboplatin and 5-fluorouracil, which are used in combination with radiotherapy [286]. Notably, compounds **2** and **3** are more cytotoxic to cancer cells than for normal HET-1A cells. Compound **2** is four times and **3** seven times more effective towards the cancer cells compared to the normal cell line.

In conclusion, compounds **2** and **3** can be used in bioassays as fluorescent markers in living cells. The incubation times are very short and initial images can be acquired after 5–20 min. Localization studies showed that the dyes accumulate in the endoplasmic reticulum and can be used as facile ER-markers. The materials proved to be cytotoxic with a remarkable preference for cancer cells and therefore can be considered as potential candidates for cancer treatment.

3.5.2 Phenanthroline Europium(III) and Zinc(II) complexes

Lanthanide ions have especially attractive luminescence properties, emitting at a millisecond lifetime-scale and in the visible region for europium(III) and terbium(III) complexes [287-290]. These remarkable photophysical properties are key factors to make them molecules of choice for cellular imaging and diagnostics [291-294]. A variety of the different Ln(III) complexes have been synthesised, however, a major problem is their instability in aqueous media. The lanthanide ion and its neutral ligands are linked via weak coordination bonds and generally exist in equilibrium of “free ligand” $\rightleftharpoons$ “metal complex”, hence the metal complexes are prone to dissociation in the presence of solvent molecules. Disadvantageously, in some cases this can lead to an irreversible dissociation into the “free ligand” form in water [295-298]. This normally prevents the use of Ln(III) complexes as luminescent markers for bioassays. Nevertheless, only limited information is available on the real state of such metal complexes in different media.

Zinc compounds are known to be highly fluorescent and present an alternative to lanthanide complexes. Thus, many photochemical studies targeted at modelling the natural situation use more stable zinc complexes. Due to their biological relevance, technical importance and stability, they have found significant use as industrial pigments, electron transfer components, for photochemical transformations and in photobiotechnology [299].

Photophysical behaviour of compounds 5–10

We studied the stability and photophysical efficiency of europium and zinc complexes of phenanthroline derivatives. These molecules are strongly

absorbing chromophores often used as antennas for indirect excitation of metal ions, where direct excitation of a metal is inefficient. Phenanthroline itself is known to inhibit metallopeptidases and its various metal complexes have been prepared and tested as bioprobes [254, 300-306].

The europium complex **5** showed the characteristic bands of lanthanide ion emission between 670 nm and 710 nm. In the case of the zinc complex **9**, a splitting of the band at 410 nm was observed with the new maximum at 465 nm as the result of complex formation (Table 3.5.2.1).

Table 3.5.2.1 Photophysical parameters of **5** and **6** in THF and **8–10** in MeOH.

Compound	Absorption peaks (nm)	Emission peaks (nm) ^a
5	279, 325	405; 591; 617; 646; 681
6	282; 339	405; 591; 617; 650; 681
8	277; 319	400
9	283; 290; 318	560
10	294; 301	495

a. Emission spectra recorded at 280 nm excitation

In addition to the emission profile requirements, fluorescence imaging of the biomaterials requires reasonable Stokes shifts. Indeed, according to the results reported in Table 3.5.2.1, all compounds show large Stokes shifts.

The determination of the lowest staining concentration was performed in the 0.74 to 10 μ M range. We have demonstrated that complexes **5** and **7** are stable (up to 48 h) in aqueous solutions (EtOH–PBS) and it still exhibit a strong luminescence (Table 3.5.1.1). The 1 mM stock ethanolic solutions of the compounds **5** and **7** were diluted with PBS buffer and an excess of crystals were

filtered to give final concentrations of 0.74 μM for **5** and 8.2 μM for **7**. The fluorescence images were taken after 20 minutes and 1 hour incubation times at 405 nm irradiation energy and detection at 535 nm (Fig. 3.5.2.1).

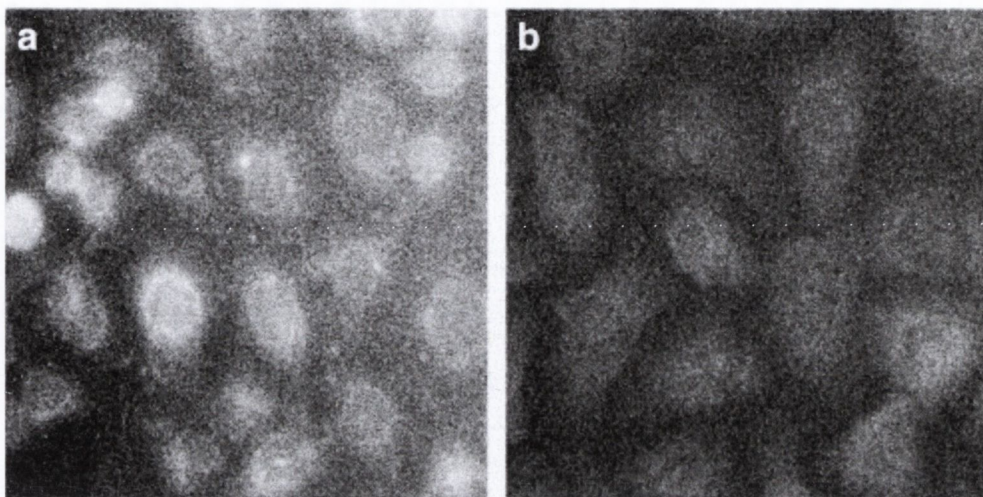


Fig. 3.5.2.1 InCell images of complexes **5** (a) and **7** (b) in OE21-fixed cells after 1 h incubation (irradiation at 405 nm and detection at 535 nm).

The zinc complexes **8–10** were less fluorescent after 1 h incubation at these concentrations, the fluorescent images were obtained for the zinc compounds **8** (1.6 μM) and **9** (0.84 μM) after 2 and 6 h incubation, respectively (Fig. 3.5.2.2). Interestingly, the fluorescence of **9** diminished with time, and no intracellular fluorescence was observed for complex **10**.

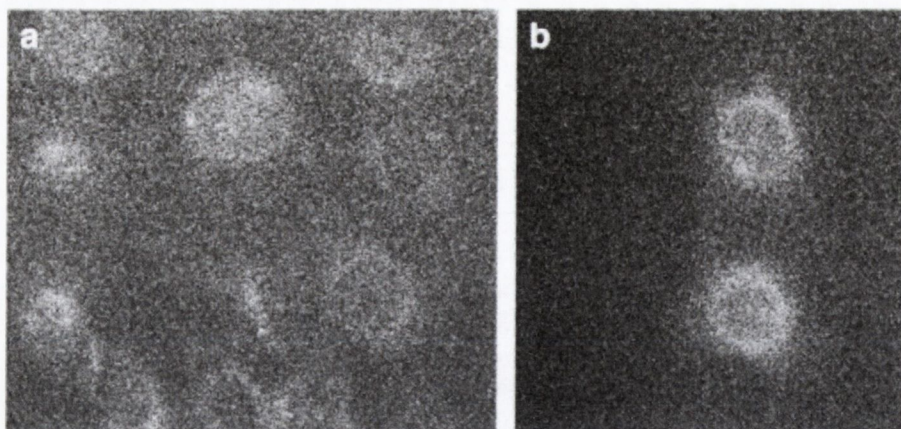


Figure 3.5.2.2 InCell images of the complexes **8** (a) and **9** (b) in EtOH–PBS pH 7.4 for OE21-fixed cells after 2 h incubation (irradiation at 405 nm and detection at 535 nm).

Overall, the europium compounds present good fluorescent intensity after 1 hour incubation and are photostable over 6 hours. In comparison, to the europium materials, the zinc complexes required longer incubation times and show lower photostability. Furthermore, fluorescence decay occurred over time as a result of photobleaching.

CHAPTER FIVE:

Conclusions and future perspectives

In vitro testing of drugs is the most important pre-clinical step on the drug discovery process as it tests for effect of potential therapeutic compounds. For many years this manual process has further increased the time needed for any new compound to reach the market. High content screening methods are designed to fail unsuitable compounds early in the drug discovery process. With automation playing a central role in these. The use of robots is now commonplace both in academic institutions and in the pharmaceutical industry and automated high content screening of compounds has, in recent years, significantly decreased the complexity and the time needed for the pre-clinical evaluation of drugs. This work focused on the development of a novel high content screening method to screen porphyrin derivatives for effect and specificity on oesophageal cancer cell lines.

The aim was to develop a quick, reliable and multi-output method for performing primary screens on large libraries of novel compounds. Simple morphological parameters that have been extensively used by toxicologists for several years were chosen deliberately [112, 193, 307, 308]. Basic area measurements such as cell area, nuclear area and cell sphericity can only change in three different ways: (i) decrease, (ii) increase, (iii) remain unchanged. The first two parameters can easily be identified using the proposed methodology and have been demonstrated to be good markers of bio-reactivity [307, 308]. If there is no change then any cytotoxicity will be detected later in the assay by assessing any changes in cell number.

The results illustrate the efficacy and the limitations of evaluating potential photosensitizing agents quantitatively using high content imaging and analysis platform technologies. This lowers the number of required manipulations steps compared to other drugs and tests. It also reduces the overall time required for analysis and evaluation, therefore, reducing experimental turnaround times. In addition, it is more cost effective than traditional testing as technological developments have made these types of equipment ever more available and inexpensive. Also, fixation allows for the storage of plates for subsequent staining (*e.g.*, organelle staining in order to clarify intracellular drug location) and for further multiplexing. Storage of images allows for re-interrogation in the future, should other questions arise. It is also important to note that these studies now allow the development of an automated high throughput screening protocol for the evaluation, QSAR studies, and cell biological investigation of photosensitisers, which is currently ongoing.

Phenotypic screens, based solely in the change of physical characteristics of cells, have recently been identified as an effective method for failing drugs early in the research and development pipeline [307, 309]. This prevents significant financial expenditures on *in vivo* screens that would ultimately fail or, in a worst case scenario, if harmful, could lead to a total recall and withdrawal of the drug from the market. In fact, 37% of all “first-in-class” drugs discovered from 1999 to 2008 were discovered through phenotypic screens [90]. PDT development is an area mostly driven by individual research groups and not larger pharmaceutical companies with libraries. Most groups focus on small libraries of chemically closely related systems, mostly based on local experience or availability of dyes; often only with minor differences in structure from group to

group. The use of HCA for large scale library screening of PS might offer new avenues for the identification of conceptionally different PS. HCA approaches as described here can offer significant advantages for the development of new PS drugs and other sensors and imaging compounds in photomedicine in general.

The generations of large libraries of novel porphyrins rendered many compounds available for biological testing; here we tested various libraries of these novel compounds with only a handful of these exhibiting uptake by test cells and even less displayed significant phototoxicity. Our method is a rapid way to fail potential fluorescent drugs earlier in the development stage.

The use of three dimensional structures in the study of potential anticancer therapies could be the missing link between tests in monolayers and tests in animal models. There is much to be gained in testing potential therapies in 3D spheroids such as ease of generating these structures *in vitro*, it removes the need for ethical approval for early tests of potential therapies as well as being significantly cheaper than keeping a cohort of animal models for testing. Once again this would fail compounds early in the discovery process.

We were able to establish easy and consistent protocols for the generation of 3D spheroids of two oesophageal cancer cell lines. We found that spheroids are more resistant to the action of PDT than monolayers and found that both previously unknown phosphoporphyrins in no way increase the destruction of these structures.

A 3D spheroids screen for potential photosensitisers using our previously established HCS protocol will be integrated into our tests of toxicity. An increased range of concentrations tested for the various potential

photosensitisers will be used in order to account for the increased resistance to PDT by the spheroids. In order to select effective PS drugs against a variety of solid tumours.

The identified hits (compounds **4**, **8**, **14**, **15** and **27**) will be taken further into clinical development. As a first step a broader concentration of these will be tested in our 3D model of oesophageal cancer, looking for significant lower IC₅₀ in these tumour mimics. Intracellular location of these compounds will be determined in an attempt to identify which cellular organelles are involved in toxicity.

Determination of cell death (apoptosis vs. necrosis) of these compounds comparing them to Temoporfin will also be assessed. Finally we will test for any upregulation/downregulation of genes related to tumour progression when both cell monolayers and spheroids are treated with PDT using these compounds.

Only when these tests have been completed will we start devising animal model tests in order to further progress with the clinical development of our compounds. Our final aim will be to bring the most promising compound into clinical trial.

In link with our interest in HCS for the development of PDT agents we developed a library of new small fluorescent molecules and tested their suitability for fluorescent imaging. From these we could determine that only a few were suitable for imaging but toxicity would constitute a problem and this issue must be addressed by chemical modifications of the molecules. On the other hand, these are also potent protease inhibitors; in the future we will test

their suitability as co-drugs with PDT to further increase the death of cell death of oesophageal cancer.

References

1. *Photodynamic Therapy at the Cellular Level*, ed. A.B. Uzdensky. 2007: Research Signpost. 271.
2. Freeley, M., et al., *A high-content analysis toolbox permits dissection of diverse signaling pathways for T lymphocyte polarization*. J Biomol Screen, 2010. **15**(5): p. 541-55.
3. Yusuke Hiraku, K.I.K.H.S.K., *Photosensitized DNA Damage and its Protection via a Novel Mechanism*. Photochem Photobiol, 2007. **83**(1): p. 205-212.
4. Mitton, D. and R. Ackroyd, *History of photodynamic therapy in Great Britain*. Photodiagn Photodyn Ther, 2005. **2**(4): p. 239-246.
5. Kessel, D., *Photodynamic therapy: from the beginning*. Photodiagn Photodyn Ther, 2004. **1**(1): p. 3-7.
6. Mitton, D. and R. Ackroyd, *A brief overview of photodynamic therapy in Europe*. Photodiagn Photodyn Ther, 2008. **5**(2): p. 103-111.
7. Kelly, J.F., M.E. Snell, and M.C. Berenbaum, *Photodynamic destruction of human bladder carcinoma*. Br J Cancer, 1975. **31**(2): p. 237-44.
8. J. H. Ryou, S.J.L.Y.M.P.H.O.K.H.S.K., *Acne-photodynamic therapy with intra-lesional injection of 5-aminolevulinic acid*. Photodermatol, Photoimmunol Photomed, 2009. **25**(1): p. 57-58.
9. Juarranz, A., et al., *Photodynamic therapy of cancer. Basic principles and applications*. Clin Transl Oncol, 2008. **10**(3): p. 148-54.
10. Hopper, C., C. Niziol, and M. Sidhu, *The cost-effectiveness of Foscan mediated photodynamic therapy (Foscan-PDT) compared with extensive palliative surgery and palliative chemotherapy for patients with*

- advanced head and neck cancer in the UK*. Oral Oncol, 2004. **40**(4): p. 372-382.
11. Juzeniene, A., Q. Peng, and J. Moan, *Milestones in the development of photodynamic therapy and fluorescence diagnosis*. Photochem Photobiol Sci, 2007. **6**(12): p. 1234-1245.
 12. Ian J. Macdonald, T.J.D., *Basic principles of photodynamic therapy*. J Porphyrins Phthalocyanines, 2001. **5**(2): p. 105-129.
 13. Shawn, S. and T. Matthew, *Porphyrin and Phthalocyanine Photosensitizers as PDT Agents: A New Modality for the Treatment of Melanoma*. 2013.
 14. Agnez-Lima, L.F., et al., *DNA damage by singlet oxygen and cellular protective mechanisms*. Mutat Res, 2012.
 15. Moan, J. and K. Berg, *The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen*. Photochem Photobiol, 1991. **53**(4): p. 549-53.
 16. Stupáková, V., et al., *Photodynamic effect of hypericin in primary cultures of human umbilical endothelial cells and glioma cell lines*. Phytother Res, 2009. **23**(1): p. 827-832.
 17. Nyst, H.J., et al., *Is photodynamic therapy a good alternative to surgery and radiotherapy in the treatment of head and neck cancer?* Photodiagn Photodyn Ther, 2009. **6**(1): p. 3-11.
 18. Wang, J.-l., et al., *Combination of Targeted PDT and Anti-VEGF Therapy for Rat CNV by RGD-Modified Liposomal Photocyanine and Sorafenib*. Invest Ophthalmol Visual Sci, 2013. **54**(13): p. 7983-7989.

19. Broekgaarden, M., et al., *Development and in Vitro Proof-of-Concept of Interstitially Targeted Zinc-Phthalocyanine Liposomes for Photodynamic Therapy*. Curr Med Chem, 2013.
20. Sibani, S.A., et al., *Photosensitiser delivery for photodynamic therapy. Part 2: systemic carrier platforms*. Expert Opin Drug Delivery, 2008. **5**(11): p. 1241-1254.
21. Seshadri, M., et al., *Tumor Vascular Response to Photodynamic Therapy and the Antivascular Agent 5,6-Dimethylxanthenone-4-Acetic Acid: Implications for Combination Therapy*. Clin Cancer Res, 2005. **11**(11): p. 4241-4250.
22. Castano, A.P., P. Mroz, and M.R. Hamblin, *Photodynamic therapy and anti-tumour immunity*. Nat Rev Cancer, 2006. **6**(7): p. 535-545.
23. Firczuk, M., D. Nowis, and J. Golab, *PDT-induced inflammatory and host responses*. Photochem Photobio Sci, 2011. **10**(5): p. 653-663.
24. Pizova, K., et al., *Photodynamic therapy for enhancing antitumour immunity*. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2012. **156**(2): p. 93-102.
25. Chaabane, W., et al., *Autophagy, apoptosis, mitoptosis and necrosis: interdependence between those pathways and effects on cancer*. Arch Immunol Ther Exp (Warsz), 2013. **61**(1): p. 43-58.
26. Mang, T.S., *Lasers and light sources for PDT: past, present and future*. Photodiagn Photodyn Ther, 2004. **1**(1): p. 43-48.
27. OCF. *Cancer of the oesophagus*. [cited November 2013]; Available from: http://www.lollipopday.com/oc_info.html.

28. Ireland, N.c.r. *Cancer Trends: Cancers of the oesophagus and stomach* 2011 [cited November 2013]; Available from:
<http://www.ncri.ie/sites/ncri/files/pubs/CancerTrendsNo.8-CancersoftheOesophagusandStomach.pdf>.
29. Ferlay, J., et al., *Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012*. Eur J Cancer, 2013. **49**(6): p. 1374-1403.
30. Robertson, L., *A New Horizon: Recommendations and Treatment Guidelines for Barrett's Esophagus*. Gastroentero Nursing, 2009. **32**(3): p. 202-210 10.1097/SGA.0b013e3181a93f7a.
31. Spechler, S.J., *Barrett esophagus and risk of esophageal cancer: a clinical review*. Jama, 2013. **310**(6): p. 627-36.
32. Agarwal, A., et al., *Role of epigenetic alterations in the pathogenesis of Barrett's esophagus and esophageal adenocarcinoma*. Int J Clin Exp Pathol, 2012. **5**(5): p. 382-96.
33. Milind, R. and S.E. Attwood, *Natural history of Barrett's esophagus*. World J Gastroenterol, 2012. **18**(27): p. 3483-91.
34. Agarwal, A., et al., *Role of epigenetic alterations in the pathogenesis of Barrett's esophagus and esophageal adenocarcinoma*. Int J Clin Exp Pathol, 2012. **5**(5): p. 382-396.
35. IrishCancerSociety. *Cancer information*. [cited November 2013]; Available from: <http://www.cancer.ie/cancerInfo/cancers.php>.
36. Pennathur, A., et al., *Oesophageal carcinoma*. Lancet, 2013. **381**(9864): p. 400-412.

37. Hamblin, M.R. and Y.-Y. Huang, *Handbook of Photomedicine*. 2013: CRC Press.
38. Varghese, T.K., et al., *The Society of Thoracic Surgeons Guidelines on the Diagnosis and Staging of Patients With Esophageal Cancer*. Ann Thorac Surg, 2013. **96**(1): p. 346-356.
39. Kawakubo, H., H. Takeuchi, and Y. Kitagawa, *Current Status and Future Perspectives on Minimally Invasive Esophagectomy*. Korean J Thorac Cardiovasc Surg, 2013. **46**(4): p. 241-248.
40. Bharat, A. and T. Crabtree, *Management of Advanced-Stage Operable Esophageal Cancer*. Surg Clin North Am, 2012. **92**(5): p. 1179-1197.
41. Sundelöf, M., J. Lagergren, and W. Ye, *Patient demographics and lifestyle factors influencing long-term survival of oesophageal cancer and gastric cardia cancer in a nationwide study in Sweden*. Eur J Cancer, 2008. **44**(11): p. 1566-1571.
42. McCann, P., et al., *The safety and effectiveness of endoscopic and non-endoscopic approaches to the management of early esophageal cancer: A systematic review*. Cancer Treat Rev, 2011. **37**(1): p. 11-62.
43. Morrow, D.J., et al., *Pathogenesis of Barrett's esophagus: Bile acids inhibit the Notch signaling pathway with induction of CDX2 gene expression in human esophageal cells*. Surgery, 2009. **146**(4): p. 714-722.
44. Moghissi, K., et al., *Photofrin PDT for early stage oesophageal cancer: Long term results in 40 patients and literature review*. Photodiagn Photodyn Ther, 2009. 6(3-4): p159-66.

45. Califano, J., et al., *Second Esophageal Tumors in Patients with Head and Neck Squamous Cell Carcinoma: An Assessment of Clonal Relationships*. Clin Cancer Res, 1999. **5**(7): p. 1862-1867.
46. Watanabe, A., et al., *Head and neck cancer associated with esophageal cancer*. Auris Nasus Larynx, 2007. **34**(2): p. 207-211.
47. Brancalion, L. and H. Moseley, *Laser and Non-laser Light Sources for Photodynamic Therapy*. Lasers Med Sci, 2002. **17**(3): p. 173-186.
48. Chatterjee, D.K., L.S. Fong, and Y. Zhang, *Nanoparticles in photodynamic therapy: An emerging paradigm*. Adv Drug Deliv Rev, 2008. **60**(15): p. 1627-1637.
49. Asta, J. and M. Johan, *The history of PDT in Norway: Part one: Identification of basic mechanisms of general PDT*. Photodiagn Photodyn Ther, 2007. **4**(1): p. 3-11.
50. Navarro, F.P., et al., *Preparation and characterization of mTHPC-loaded solid lipid nanoparticles for photodynamic therapy*. J Photochem Photobiol, B, 2014. **130**(0): p. 161-169.
51. Agostinis, P., et al., *Photodynamic therapy of cancer: an update*. CA Cancer J Clin, 2011. **61**(4): p. 250-281.
52. Piffaretti, F., et al., *Correlation between Protoporphyrin IX Fluorescence Intensity, Photobleaching, Pain and Clinical Outcome of Actinic Keratosis Treated by Photodynamic Therapy*. Dermatology, 2013. **227**(3): p. 214-25.
53. Nyst, H.J., et al., *Temoporfin mediated photodynamic therapy in patients with local persistent and recurrent nasopharyngeal carcinoma after curative radiotherapy: a feasibility study*. Photodiagn Photodyn Ther, 2012. **9**(3): p. 274-81.

54. Jerjes, W., et al., *Interstitial PDT for vascular anomalies*. Lasers Surg Med, 2011. **43**(5): p. 357-65.
55. Indrasari, S.R., et al., *Remarkable response to photodynamic therapy in residual T4N0M0 nasopharyngeal carcinoma: a case report*. Photodiagn Photodyn Ther, 2012. **9**(4): p. 319-20.
56. Vyslouzilova, D., et al., *[Photodynamic therapy with Verteporfin in treatment of wet form ARMD - long term results]*. Cesk Slov Oftalmol, 2012. **68**(3): p. 98-101.
57. Seidel, G., et al., *Combination treatment of photodynamic therapy with verteporfin and intravitreal ranibizumab in patients with retinal angiomatous proliferation*. Acta Ophthalmol, 2013. **91**(6): p. e482-5.
58. Tsuchihashi, T., et al., *Five-year results of photodynamic therapy with verteporfin for Japanese patients with neovascular age-related macular degeneration*. Clin Ophthalmol, 2013. **7**: p. 615-20.
59. Buinauskaite, E., et al., *Topical photodynamic therapy of actinic keratoses with 5-aminolevulinic acid: Randomized controlled trial with six months follow-up*. J Dermatolog Treat, 2013.
60. Tanaka, N., et al., *Comparative study for the effect of photodynamic therapy, imiquimod immunotherapy and combination of both therapies on 40 lesions of actinic keratosis in Japanese patients*. J Dermatol, 2013. **40**(12): p. 962-7.
61. Cai, H., et al., *Photodynamic therapy for facial actinic keratosis: a clinical and histological study in Chinese patients*. Photodiagn Photodyn Ther, 2013. **10**(3): p. 260-5.

62. Casie Chetty, N., B. Hemmant, and A.M. Skellett, *Periocular photodynamic therapy for squamous intra-epidermal carcinoma*. J Dermatolog Treat, 2013.
63. Barge, J., et al., *Correlations between photoactivable porphyrins' fluorescence, erythema and the pain induced by PDT on normal skin using ALA-derivatives*. Photodiagn Photodyn Ther, 2013. **10**(4): p. 683-93.
64. Tzogani, K., et al., *The European Medicines Agency approval of 5-aminolaevulinic acid (Ameluz) for the treatment of actinic keratosis of mild to moderate intensity on the face and scalp: Summary of the scientific assessment of the Committee for Medicinal Products for Human Use*. J Dermatolog Treat, 2014. **25**(5): p. 371-4.
65. Davilla, M., et al., *Photodynamic therapy for ablation of Barrett's esophagus*.
66. FDA. *Photofrin approval history*. [cited November 2013]; Available from:
http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Label_ApprovalHistory#apphist.
67. EMEA. *EPARs for authorised medicinal products for human use*. [cited November 2013]; Available from:
<http://www.emea.europa.eu/humandocs/Humans/EPAR/foscan/foscan.htm>.
68. Ericson, M.B., A.M. Wennberg, and O. Larko, *Review of photodynamic therapy in actinic keratosis and basal cell carcinoma*. Ther Clin Risk Manag, 2008. **4**(1): p. 1-9.

69. Huang, Z., *A review of progress in clinical photodynamic therapy*. Technol Cancer Res Treat, 2005. **4**(3): p. 283-93.
70. Zhu, T.C. and J.C. Finlay, *Prostate PDT dosimetry*. Photodiagn Photodyn Ther, 2006. **3**(4): p. 234-246.
71. Robinson, D.J., et al., *Fluorescence Photobleaching of ALA-induced Protoporphyrin IX during Photodynamic Therapy of Normal Hairless Mouse Skin: The Effect of Light Dose and Irradiance and the Resulting Biological Effect*. Photochem Photobio, 1998. **67**(1): p. 140-149.
72. Henderson, B.W., et al., *Photofrin photodynamic therapy can significantly deplete or preserve oxygenation in human basal cell carcinomas during treatment, depending on fluence rate*. Cancer Res, 2000. **60**(3): p. 525-529.
73. Wang, H.-W., et al., *Effect of Photosensitizer Dose on Fluence Rate Responses to Photodynamic Therapy†*. Photochem Photobio, 2007. **83**(5): p. 1040-1048.
74. Grosjean, P., et al., *Clinical photodynamic therapy for superficial cancer in the oesophagus and the bronchi: 514 nm compared with 630 nm light irradiation after sensitization with Photofrin II*. Br J Cancer, 1998. **77**(11): p. 1989-95.
75. Triesscheijn, M., et al., *Photodynamic Therapy in Oncology*. Oncologist, 2006. **11**(9): p. 1034-1044.
76. Gold, M.H., *Photodynamic therapy with lasers and intense pulsed light*. Facial Plast Surg Clin North Am, 2007. **15**(2): p. 145-60, v.
77. Shaaban, D., Z. Abdel-Samad, and M. El-Khalawany, *Photodynamic therapy with intralesional 5-aminolevulinic acid and intense pulsed light*

- versus intense pulsed light alone in the treatment of acne vulgaris: a comparative study.* Dermatol Ther, 2012. **25**(1): p. 86-91.
78. Yoon, I., J.Z. Li, and Y.K. Shim, *Advance in Photosensitizers and Light Delivery for Photodynamic Therapy.* Clin Endosc, 2013. **46**(1): p. 7-23.
79. Dragunow, M., *High-content analysis in neuroscience.* Nat Rev Neurosci, 2008. **9**(10): p. 779-788.
80. Dove, A., *Screening for content - the evolution of high throughput.* Nat Biotech, 2003. **21**(8): p. 859-864.
81. Abraham, V.C., D.L. Taylor, and J.R. Haskins, *High content screening applied to large-scale cell biology.* Trends Biotechnol, 2004. **22**(1): p. 15-22.
82. Johnston, P.A. and P.A. Johnston, *Cellular platforms for HTS: three case studies.* Drug Discovery Today, 2002. **7**(6): p. 353-363.
83. Smellie, A., C.J. Wilson, and S.C. Ng, *Visualization and Interpretation of High Content Screening Data.* J Chem Inf Model, 2005. **46**(1): p. 201-207.
84. Swinney, D.C. and J. Anthony, *How were new medicines discovered?* Nat Rev Drug Discov, 2011. **10**(7): p. 507-519.
85. Reisen, F., et al., *Benchmarking of multivariate similarity measures for high-content screening fingerprints in phenotypic drug discovery.* J Biomol Screen, 2013. **18**(10): p. 1284-97.
86. Zanella, F., J.B. Lorens, and W. Link, *High content screening: seeing is believing.* Trends in Biotechnol, 2010. **28**(5): p. 237-245.
87. Latt, S.A., et al., *Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence.* J Histochem Cytochem, 1975. **23**(7): p. 493-505.

88. Xu, J.J., D. Diaz, and P.J. O'Brien, *Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential*. Chem Biol Interact, 2004. **150**(1): p. 115-128.
89. Abraham, V.C., et al., *Application of a high-content multiparameter cytotoxicity assay to prioritize compounds based on toxicity potential in humans*. J Biomol Screen, 2008. **13**(6): p. 527-537.
90. Swinney, D.C. and J. Anthony, *How were new medicines discovered?* Nat Rev Drug Discov, 2011. **10**(7): p. 507-19.
91. Bickle, M., *The beautiful cell: high-content screening in drug discovery*. Anal Bioanal Chem, 2010. **398**(1): p. 219-226.
92. Moan, J. and K. Berg, *The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen*. Photochem Photobiol, 1991. **53**(4): p. 549-553.
93. Atif, M., *Fluorescence photobleaching dynamics of meso tetra hydroxy phenyl chlorin (mTHPC)*. Laser Phys Lett, 2012. **9**(5): p. 387.
94. Gouterman, M., *Spectra of porphyrins*. J Mol Spectrosc, 1961. **6**: p. 138-163.
95. Yu, Z., et al., *Photodynamic therapy on human pancreatic cancer cells: an in vitro study of main influencing factors*. Biol Biomed Rep, 2011. **2**(1).
96. Tim, M., *Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays*. J Immunol Methods, 1983. **65**(1-2): p. 55-63.
97. Tada, H., et al., *An improved colorimetric assay for interleukin 2*. J Immunol Methods, 1986. **93**(2): p. 157-165.

98. Sherifa, G., et al., *Functional characterization of Fospeg, and its impact on cell cycle upon PDT of Huh7 hepatocellular carcinoma cell model*. Photodiagn Photodyn Ther, 2013. **10**(1): p.87-94.
99. Amin, R.M., et al., *Evaluation of photodynamic treatment using aluminum phthalocyanine tetrasulfonate chloride as a photosensitizer: new approach*. Photochem Photobiol Sci, 2012. **11**(7): p. 1156-1163.
100. Celli, J.P., et al., *Verteporfin-based photodynamic therapy overcomes gemcitabine insensitivity in a panel of pancreatic cancer cell lines*. Lasers Surg Med, 2011. **43**(7): p. 565-74.
101. Grebeňová, D., et al., *Selective destruction of leukaemic cells by photo-activation of 5-aminolaevulinic acid-induced protoporphyrin-IX*. J Photochem Photobiol, B, 1998. **47**(1): p. 74-81.
102. Gilaberte, Y., et al., *Flow cytometry study of the role of superoxide anion and hydrogen peroxide in cellular photodestruction with 5-aminolevulinic acid-induced protoporphyrin IX*. Photodermatol, Photoimmunol Photomed, 1997. **13**(1-2): p. 43-49.
103. Zhu, J., et al. *Research off ALA combined with HpD-PDT which induced S180 ascitic tumor cells, death or apoptosis on cytology*. 2005.
104. Tirapelli, L.F., et al., *Apoptosis in glioma cells treated with PDT*. Photomed Laser Surg, 2011. **29**(5): p. 305-9.
105. Leunig, A., et al., *Effect of photodynamic treatment of human endothelial cells on cell volume and cell viability*. Int J Oncol, 1996. **8**(6): p. 1217-21.
106. Saczko, J., et al., *The influence of photodynamic therapy on apoptosis in human melanoma cell line*. Folia Histochem Cytobiol, 2005. **43**(3): p. 129-32.

107. Repetto, G., A. del Peso, and J.L. Zurita, *Neutral red uptake assay for the estimation of cell viability/cytotoxicity*. Nat. Protocols, 2008. **3**(7): p. 1125-1131.
108. Sharma, S., et al., *Pharmacokinetics and phototoxicity of purpurin-18 in human colon carcinoma cells using liposomes as delivery vehicles*. Cancer Chemother Pharmacol, 2006. **57**(4): p. 500-6.
109. Mitra, S., O. Mironov, and T.H. Foster, *Confocal fluorescence imaging enables noninvasive quantitative assessment of host cell populations in vivo following photodynamic therapy*. Theranostics, 2012. **2**(9): p. 840-9.
110. Radzi, R., et al., *Morphological study in B16F10 murine melanoma cells after photodynamic hyperthermal therapy with indocyanine green (ICG)*. J Vet Med Sci, 2012. **74**(4): p. 465-72.
111. Amin, R.M., et al., *Evaluation of photodynamic treatment using aluminum phthalocyanine tetrasulfonate chloride as a photosensitizer: new approach*. Photochem Photobiol Sci, 2012. **11**(7): p. 1156-63.
112. Haney, S.A., et al., *High-content screening moves to the front of the line*. Drug Discov Today, 2006. **11**(19-20): p. 889-894.
113. Lieggi, N.T., A. Edvardsson, and P.J. O'Brien, *Translation of novel anti-cancer cytotoxicity biomarkers detected with high content analysis from an in vitro predictive model to an in vivo cell model*. Toxicol In Vitro, 2010. **24**(8): p. 2063-2071.
114. Fennema, E., et al., *Spheroid culture as a tool for creating 3D complex tissues*. Trends Biotechnol, 2013. **31**(2): p. 108-115.

115. Pampaloni, F., E.G. Reynaud, and E.H.K. Stelzer, *The third dimension bridges the gap between cell culture and live tissue*. Nat Rev Mol Cell Biol, 2007. **8**(10): p. 839-845.
116. Hongisto, V., et al., *High-Throughput 3D Screening Reveals Differences in Drug Sensitivities between Culture Models of JIMT1 Breast Cancer Cells*. PLoS One, 2013. **8**(10): p. e77232.
117. Marx, V., *Cell culture: A better brew*. Nature, 2013. **496**(7444): p. 253-258.
118. Ivascu, A. and M. Kubbies, *Rapid Generation of Single-Tumor Spheroids for High-Throughput Cell Function and Toxicity Analysis*. J Biomol Screen, 2006. **11**(8): p. 922-932.
119. Truong, H.H., et al., *Automated microinjection of cell-polymer suspensions in 3D ECM scaffolds for high-throughput quantitative cancer invasion screens*. Biomaterials, 2012. **33**(1): p. 181-188.
120. Asthana, A. and W.S. Kisaalita, *Biophysical microenvironment and 3D culture physiological relevance*. Drug Discov Today, 2013. **18**(11–12): p. 533-540.
121. Klein, O.J., Y.K. Jung, and C.L. Evans, *Longitudinal, quantitative monitoring of therapeutic response in 3D in vitro tumor models with OCT for high-content therapeutic screening*. Methods, 2013. In press
122. Kim, J.B., *Three-dimensional tissue culture models in cancer biology*. Semin Cancer Biol, 2005. **15**(5): p. 365-377.
123. Patil, S.A., et al., *Novel approaches to glioma drug design and drug screening*. Expert Opin Drug Discov, 2013. **8**(9): p. 1135-1151.
124. Denison, T.A. and Y.H. Bae, *Tumor heterogeneity and its implication for drug delivery*. J Controlled Release, 2012. **164**(2): p. 187-191.

125. Henderson, B.W. and V.H. Fingar, *Relationship of tumor hypoxia and response to photodynamic treatment in an experimental mouse tumor*. Cancer Res, 1987. **47**(12): p. 3110-4.
126. Wyld, L., M.W. Reed, and N.J. Brown, *The influence of hypoxia and pH on aminolaevulinic acid-induced photodynamic therapy in bladder cancer cells in vitro*. Br J Cancer, 1998. **77**(10): p. 1621-7.
127. Sitnik, T.M., J.A. Hampton, and B.W. Henderson, *Reduction of tumour oxygenation during and after photodynamic therapy in vivo: effects of fluence rate*. Br J Cancer, 1998. **77**(9): p. 1386-94.
128. Longati, P., et al., *3D pancreatic carcinoma spheroids induce a matrix-rich, chemoresistant phenotype offering a better model for drug testing*. BMC Cancer, 2013. **13**(1): p. 95.
129. Kunz-Schughart, L.A., et al., *The use of 3-D cultures for high-throughput screening: the multicellular spheroid model*. J Biomol Screen, 2004. **9**(4): p. 273-85.
130. Friedrich, J., R. Ebner, and L.A. Kunz-Schughart, *Experimental anti-tumor therapy in 3-D: spheroids--old hat or new challenge?* Int J Radiat Biol, 2007. **83**(11-12): p. 849-71.
131. Ho, W.J., et al., *Incorporation of multicellular spheroids into 3-D polymeric scaffolds provides an improved tumor model for screening anticancer drugs*. Cancer Sci, 2010. **101**(12): p. 2637-2643.
132. Sergeeva, N.N., et al., *Synthesis and evaluation of the europiumIII and zincII complexes as luminescent bioprobes in high content cell-imaging analysis*. J Inorg Biochem, 2011. **105**(12): p. 1589-1595.
133. Cao, H.S.T., et al., *Imaging of the interaction of cancer cells and the lymphatic system*. Adv Drug Deliv Rev, 2011. **63**(10-11): p. 886-889.

134. Suhling, K., P.M.W. French, and D. Phillips, *Time-resolved fluorescence microscopy*. Photochem Photobiol Sci, 2005. **4**(1): p. 13-22.
135. Kobayashi, H., et al., *New Strategies for Fluorescent Probe Design in Medical Diagnostic Imaging*. Chem Rev, 2009. **110**(5): p. 2620-2640.
136. Yen, S.K., et al., *Design and Synthesis of Polymer-Functionalized NIR Fluorescent Dyes–Magnetic Nanoparticles for Bioimaging*. ACS Nano, 2013. **7**(8): p. 6796-6805.
137. Lakowicz, J.R., *Principles of fluorescence spectroscopy*. 2009: Springer.
138. Wysocki, L.M. and L.D. Lavis, *Advances in the chemistry of small molecule fluorescent probes*. Curr Opin Chem Biol, 2011. **15**(6): p. 752-759.
139. Drummen, G., *Fluorescent Probes and Fluorescence (Microscopy) Techniques — Illuminating Biological and Biomedical Research*. Molecules, 2012. **17**(12): p. 14067-14090.
140. Vaz, G.M., et al., *High content screening as high quality assay for biological evaluation of photosensitizers in vitro*. PLoS One, 2013. **8**(7): p. e70653.
141. Paszko, E., et al., *Transferrin conjugation does not increase the efficiency of liposomal Foscan during in vitro photodynamic therapy of oesophageal cancer*. Eur J Pharm Sci, 2013. **48**(1–2): p. 202-210.
142. Hadjantonakis, A.K., S. Macmaster, and A. Nagy, *Embryonic stem cells and mice expressing different GFP variants for multiple non-invasive reporter usage within a single animal*. BMC Biotechnol, 2002. **2**: p. 11.
143. Liao, H., et al., *An integrated diagnosis and therapeutic system using intra-operative 5-aminolevulinic-acid-induced fluorescence guided*

- robotic laser ablation for precision neurosurgery*. *Med Image Anal*, 2012. **16**(3): p. 754-766.
144. Haney, S.A., et al., *An Introduction to High Content Screening and Analysis Techniques: Practical Advice and Examples*. 2011: John Wiley & Sons, Incorporated.
 145. Zhang, X.H., et al., *Synthesis, spectral properties of cell-permeant dimethine cyanine dyes and their application as fluorescent probes in living cell imaging and flow cytometry*. *Dyes and Pigm*, 2014. **100**(0): p. 232-240.
 146. Zhang, X., et al., *Fabrication of water-dispersible and biocompatible red fluorescent organic nanoparticles via PEGylation of aggregate induced emission enhancement dye and their cell imaging applications*. *Colloids Surf B Biointerfaces*, 2014. **113**(0): p. 435-441.
 147. Zhao, H., et al., *Rationale for the real-time and dynamic cell death assays using propidium iodide*. *Cytometry A*, 2010. **77**(4): p. 399-405.
 148. Wang, M., V.M. Berthoud, and E.C. Beyer, *Connexin43 increases the sensitivity of prostate cancer cells to TNF α -induced apoptosis*. *J Cell Sci*, 2007. **120**(Pt 2): p. 320-9.
 149. Andreu-Fernandez, V., et al., *BH3-Mimetics- and Cisplatin-Induced Cell Death Proceeds through Different Pathways Depending on the Availability of Death-Related Cellular Components*. *PLoS One*, 2013. **8**(2).
 150. Tassi, E., et al., *Role of Apollon in Human Melanoma Resistance to Antitumor Agents That Activate the Intrinsic or the Extrinsic Apoptosis Pathways*. *Clin Cancer Res*, 2012. **18**(12): p. 3316-27.

151. Marciniak, A., et al., *Mouse Pancreas Tissue Slice Culture Facilitates Long-Term Studies of Exocrine and Endocrine Cell Physiology in situ*. PLoS One, 2013. **8**(11).
152. Canton, D.A., et al., *The Role of CKIP-1 in Cell Morphology Depends on Its Interaction with Actin-capping Protein**. J Biol Chem, 2006. **281**(47): p. 36347-59.
153. Lin, G., et al., *Improved Penile Histology by Phalloidin Stain: Circular and Longitudinal Cavernous Smooth Muscles, Dual-Endothelium Arteries, and Erectile Dysfunction-Associated Changes*. Urology, 2011. **78**(4): p. 970.e1-8.
154. Melan, M.A., *Use of fluorochrome-tagged taxol to produce fluorescent microtubules in solution*. Biotechniques, 1998. **25**(2): p. 188-92.
155. Martin, C., et al., *The expression of P-glycoprotein does influence the distribution of novel fluorescent compounds in solid tumour models*. Br J Cancer, 2003. **89**(8): p. 1581-9.
156. von Knobloch, R., et al., *High frequency of serum DNA alterations in renal cell carcinoma detected by fluorescent microsatellite analysis*. Int J Cancer Suppl, 2002. **98**(6): p. 889-894.
157. von Knobloch, R., et al., *Molecular Serological Detection of DNA Alterations in Transitional Cell Carcinoma Is Highly Sensitive and Stage Independent*. Clin Cancer Res, 2004. **10**(3): p. 988-993.
158. Bayani, J. and J.A. Squire, *Fluorescence in situ hybridization (FISH)*. Curr Protoc Cell Biol, 2004: p. 22.4. 1-22.4. 52.
159. Egozcue, J., J. Blanco, and F. Vidal, *Chromosome studies in human sperm nuclei using fluorescence in-situ hybridization (FISH)*. Hum Reprod Update, 1997. **3**(5): p. 441-452.

160. Esteves, S.C., *A clinical appraisal of the genetic basis in unexplained male infertility*. J Hum Reprod Sci, 2013. **6**(3): p. 176-182.
161. Ward, B.E., et al., *Rapid prenatal diagnosis of chromosomal aneuploidies by fluorescence in situ hybridization: clinical experience with 4,500 specimens*. Am J Hum Genet, 1993. **52**(5): p. 854.
162. Kempen, J.H., et al., *Fluorescein Angiography versus Optical Coherence Tomography for Diagnosis of Uveitic Macular Edema*. Ophthalmology, 2013. **120**(9): p. 1852-1859.
163. HPA. *General Cell Collection Detail: OE21*. November 2013 2009]; Available from: http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=96062201&collection=ecacc_gc.
164. J. C. Rockett, K.L., S. J. Darnton, A. G. Morris, and H. R. Matthews, *Five newly established oesophageal carcinoma cell lines: phenotypic and immunological characterization*. Br J Cancer, 1997. **75**(2): p. 258-263.
165. HPA. *General Cell Collection Detail: OE33*. November 2013]; Available from: http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=96070808&collection=ecacc_gc.
166. Nasser, A., et al., *Characterization of cell lines established from human gastric-esophageal adenocarcinomas: Biologic phenotype and invasion potential*. Cancer, 1993. **72**(3): p. 649-657.
167. Stoner, G.D., et al., *Establishment and characterization of SV40 T-antigen immortalized human esophageal epithelial cells*. Cancer Res, 1991. **51**(1): p. 365-71.

168. Bonnett, R., et al., *Hydroporphyrins of the meso-tetra(hydroxyphenyl)porphyrin series as tumour photosensitizers*. Biochem J, 1989. **261**(1): p. 277-80.
169. Feest, C., *Photophysical Characterisation and in vitro testing of novel Tetraphenylporphyrin derivative with respect to their application in Photodynamic Therapy*, in *Mathematisch-Naturwissenschaftliche Fakultät I Institut für Physik*. 2009, Humboldt Universität Berlin.
170. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays*. J Immunol Methods, 1983. **65**(1-2): p. 55-63.
171. Ramirez, C.N., et al., *Validation of a high-content screening assay using whole-well imaging of transformed phenotypes*. Assay Drug Dev Technol, 2011. **9**(3): p. 247-261.
172. Barbaric, I., et al., *High-content screening for chemical modulators of embryonal carcinoma cell differentiation and survival*. J Biomol Screen, 2011. **16**(6): p. 603-617.
173. Frisoli, J.K., et al., *Pharmacokinetics of a fluorescent drug using laser-induced fluorescence*. Cancer Res, 1993. **53**(24): p. 5954-5961.
174. Martin, C., et al., *The expression of P-glycoprotein does influence the distribution of novel fluorescent compounds in solid tumour models*. Br J Cancer, 2003. **89**(8): p. 1581-1589.
175. Kalayda, G.V., et al., *Dinuclear platinum anticancer complexes with fluorescent N,N'-bis(aminoalkyl)-1,4-diaminoanthraquinones: cellular processing in two cisplatin-resistant cell lines reflects the differences in their resistance profiles*. J Biol Inorg Chem, 2005. **10**(3): p. 305-315.

176. Li, Y., et al., *Amplification of LAPTM4B and YWHAZ contributes to chemotherapy resistance and recurrence of breast cancer*. Nat Med, 2010. **16**(2): p. 214-218.
177. Carmichael, J., et al., *Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity*. Cancer Res, 1987. **47**(4): p. 943-946.
178. Morgan, D.L., *Tetrazolium (MTT) Assay for Cellular Viability and Activity*, in *Polyamine Protocols*, D.L. Morgan, Editor. 1998, Humana Press. p. 179-184.
179. Tada, H., et al., *An improved colorimetric assay for interleukin 2*. J Immunol Methods, 1986. **93**(2): p. 157-65.
180. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays*. J Immunol Methods, 1983. **65**(1-2): p. 55-63.
181. Hamid, R., et al., *Comparison of alamar blue and MTT assays for high through-put screening*. Toxicol In Vitro, 2004. **18**(5): p. 703-710.
182. Kiesslich, T., et al., *Comparative characterization of the efficiency and cellular pharmacokinetics of Foscan- and Foslip-based photodynamic treatment in human biliary tract cancer cell lines*. Photochem Photobiol Sci, 2007. **6**(6): p. 619-627.
183. Senge, M.O. and J.C. Brandt, *Temoporfin (Foscan®), 5,10,15,20-tetra(m-hydroxyphenyl)chlorin)--a second-generation photosensitizer*. Photochem Photobiol, 2011. **87**(6): p. 1240-96.
184. Mohamed, B.M., et al., *Activation of stress-related signalling pathway in human cells upon SiO₂ nanoparticles exposure as an early indicator of cytotoxicity*. J Nanobiotechnology, 2011. **9**: p. 29.

185. Bortner, C.D. and J.A. Cidlowski, *Apoptotic volume decrease and the incredible shrinking cell*. Cell Death Differ, 2002. **9**(12): p. 1307-1310.
186. Teiten, M.H., et al., *Endoplasmic reticulum and Golgi apparatus are the preferential sites of Foscan[reg] localisation in cultured tumour cells*. Br J Cancer, 2003. **88**(1): p. 146-152.
187. Lassalle, H.-P., et al., *Fluorescence imaging of Foscan® and Foslip in the plasma membrane and in whole cells*. J Photochem Photobiol, B, 2008. **92**(1): p. 47-53.
188. Petri, A., et al., *Comparative characterization of the cellular uptake and photodynamic efficiency of Foscan® and Fospeg in a human prostate cancer cell line*. Photodiagnosis Photodyn Ther, 2012. **9**(4): p. 344-354.
189. Bovis, M.J., et al., *Improved in vivo delivery of m-THPC via pegylated liposomes for use in photodynamic therapy*. J Control Release, 2012. **157**(2): p. 196-205.
190. Preuß, A., et al., *Photosensitizer loaded HSA nanoparticles II: In vitro investigations*. Int J Pharm, 2011. **404**(1-2): p. 308-316.
191. Márquez-Ramírez, S.G., et al., *Titanium dioxide nanoparticles inhibit proliferation and induce morphological changes and apoptosis in glial cells*. Toxicology, 2012. **302**(2-3): p. 146-156.
192. Berenbaum, M.C., et al., *meso-Tetra(hydroxyphenyl)porphyrins, a new class of potent tumour photosensitisers with favourable selectivity*. Br J Cancer, 1986. **54**(5): p. 717-25.
193. Nyman, E.S. and P.H. Hynninen, *Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy*. J Photochem Photobiol, B, 2004. **73**(1-2): p. 1-28.

194. Daly, R., et al., *Synthesis and Biological Evaluation of a Library of Glycoporphyrin Compounds*. Chem Eur J, 2012. **18**(46): p. 14671-14679.
195. Wiehe, A., et al., *Lead structures for applications in photodynamic therapy. Part 1: Synthesis and variation of m-THPC (Temoporfin) related amphiphilic A2BC-type porphyrins*. Tetrahedron, 2005. **61**(23): p. 5535-5564.
196. Senge, M.O., et al., *Synthesis of meso-Substituted ABCD-Type Porphyrins by Functionalization Reactions*. Eur. J. Org. Chem, 2010. **2010**(2): p. 207-207.
197. Dinarello, C.A., *Anti-inflammatory Agents: Present and Future*. Cell, 2010. **140**(6): p. 935-950.
198. Mroz, P., et al., *Cell Death Pathways in Photodynamic Therapy of Cancer*. Cancers, 2011. **3**(2): p. 2516-2539.
199. Allison, R.R. and K. Moghissi, *Photodynamic Therapy (PDT): PDT Mechanisms*. Clin Endosc, 2013. **46**(1): p. 24-29.
200. Korbélik, M., *PDT-associated host response and its role in the therapy outcome*. Lasers Surg Med, 2006. **38**(5): p. 500-508.
201. Bergner, T. and B. Przybilla, *Photosensitization caused by ibuprofen*. J Am Acad Dermatol, 1992. **26**(1): p. 114-116.
202. Varki, A., *Biological roles of oligosaccharides: all of the theories are correct*. Glycobiology, 1993. **3**(2): p. 97-130.
203. Esko, J., K. Kimata, and U. Lindahl, *Essentials of glycobiology*. 2009, Cold Spring Harbor Laboratory Press, New York.
204. Dwek, R.A., *Glycobiology: Toward understanding the function of sugars*. Chemical Revi, 1996. **96**(2): p. 683-720.

205. Gamblin, D.P., E.M. Scanlan, and B.G. Davis, *Glycoprotein synthesis: an update*. Chem Rev, 2009. **109**(1): p. 131-63.
206. Sharon, N., *Carbohydrates as future anti-adhesion drugs for infectious diseases*. Biochim Biophys Acta, 2006. **1760**(4): p. 527-37.
207. Shukla, R.K. and A. Tiwari, *Carbohydrate Molecules: An Expanding Horizon in Drug Delivery and Biomedicine*. Crit Rev Ther Drug Carrier Syst, 2011. **28**(3): p. 255-292.
208. Hong, S.Y., et al., *Filled and glycosylated carbon nanotubes for in vivo radioemitter localization and imaging*. Nat Mat, 2010. **9**(6): p. 485-490.
209. Vliegenthart, J.F., *Carbohydrate based vaccines*. FEBS Lett, 2006. **580**(12): p. 2945-50.
210. Lis, H. and N. Sharon, *Lectins: Carbohydrate-specific proteins that mediate cellular recognition*. Chem Rev, 1998. **98**(2): p. 637-674.
211. Sharon, N. and I. Ofek, 3.28 - *Microbial Lectins*, in *Comprehensive Glycoscience*, H. Kamerling, Editor. 2007, Elsevier: Oxford. p. 623-659.
212. Sverreremark, E. and C. Fernandez, *Immunogenicity of bacterial carbohydrates: cholera toxin modulates the immune response against dextran B512*. Immunol, 1997. **92**(1): p. 153-159.
213. Hakomori, S. and R. Kannagi, *Glycosphingolipids as tumor-associated and differentiation markers*. J Natl Cancer Inst, 1983. **71**(2): p. 231-51.
214. Feizi, T., *Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens*. Nature, 1985. **314**(6006): p. 53-7.
215. Fukuda, M., *Possible roles of tumor-associated carbohydrate antigens*. Cancer Res, 1996. **56**(10): p. 2237-44.

216. Monsigny, M., et al., *Characterization and biological implications of membrane lectins in tumor, lymphoid and myeloid cells*. Biochimie, 1988. **70**(11): p. 1633-49.
217. Lotan, R. and A. Raz, *LECTINS IN CANCER-CELLS*. Ann N Y Acad Sci, 1988. **551**: p. 385-398.
218. Gabius, H.J., *Detection and functions of mammalian lectins--with emphasis on membrane lectins*. Biochim Biophys Acta, 1991. **1071**(1): p. 1-18.
219. Irimura, T., et al., *Increased content of an endogenous lactose-binding lectin in human colorectal carcinoma progressed to metastatic stages*. Cancer Res, 1991. **51**(1): p. 387-93.
220. Wilson, B.C. and M.S. Patterson, *The physics, biophysics and technology of photodynamic therapy*. Phys Med Biol, 2008. **53**(9): p. R61-R109.
221. Celli, J.P., et al., *Imaging and Photodynamic Therapy: Mechanisms, Monitoring, and Optimization*. Chem Rev, 2010. **110**(5): p. 2795-2838.
222. Ris, H.B., et al., *Photodynamic therapy with chlorins for diffuse malignant mesothelioma: initial clinical results*. Br J Cancer, 1991. **64**(6): p. 1116-20.
223. Jayashankar, L., et al., *Evaluation of photodynamic activity of metalloporphyrins on human neuroblastoma cell line*. Pharmacologyonline, 2008. **1**: p. 66-77
224. Huang, Q., et al., *Zinc(II) and copper(II) complexes of β -substituted hydroxylporphyrins as tumor photosensitizers*. Bioorg Med Chem Lett, 2006. **16**(11): p. 3030-3033.
225. Hirohara, S., et al., *Synthesis and Photocytotoxicity of S-Glucosylated 5,10,15,20-Tetrakis(tetrafluorophenyl)porphyrin Metal Complexes as*

- Efficient 1O₂-Generating Glycoconjugates*. Bioconjug Chem, 2009. **20**(5): p. 944-952.
226. Byrne, A.-M., et al., *Bile acids modulate the Golgi membrane fission process via a protein kinase C η and protein kinase D-dependent pathway in colonic epithelial cells*. Carcinogenesis, 2010. **31**(4): p. 737-744.
227. Byrne, A.M., et al., *Deoxycholic acid impairs glycosylation and fucosylation processes in esophageal epithelial cells*. Glycobiology, 2012. **22**(5): p. 638-648.
228. O'Donovan, T.R., G.C. O'Sullivan, and S.L. McKenna, *Induction of autophagy by drug-resistant esophageal cancer cells promotes their survival and recovery following treatment with chemotherapeutics*. Autophagy, 2011. **7**(5): p. 509-524.
229. Garcia, G., et al., *Microwave-mediated 'click-chemistry' synthesis of glycoporphyrin derivatives and in vitro photocytotoxicity for application in photodynamic therapy*. Tetrahedron, 2011. **67**(26): p. 4924-4932.
230. Carloni, P., et al., *On the use of 1, 3-diphenylisobenzofuran (Dphf)-reactions with carbon and oxygen-centered radicals in model and natural systems*. Res. Chem. Intermed., 1993. **19**: p. 395-405.
231. Gorman, A., et al., *In vitro demonstration of the heavy-atom effect for photodynamic therapy*. J Am Chem Soc, 2004. **126**(34): p. 10619-10631.
232. Nyman, E.S. and P.H. Hynninen, *Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy*. J Photochem Photobiol, B, 2004. **73**(1-2): p. 1-28.
233. Hudson, R., H. Savoie, and R.W. Boyle, *Lipophilic cationic porphyrins as photodynamic sensitisers—Synthesis and structure–activity relationships*. Photodiagnosis Photodyn Ther, 2005. **2**(3): p. 193-196.

234. Friedrich, J., et al., *Spheroid-based drug screen: considerations and practical approach*. Nat. Protocols, 2009. **4**(3): p. 309-324.
235. Evans, C.L., et al., *Killing hypoxic cell populations in a 3D tumor model with EtNBS-PDT*. PloS one, 2011. **6**(8): p. e23434.
236. Hockel, M. and P. Vaupel, *Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects*. J Natl Cancer Inst, 2001. **93**(4): p. 266-76.
237. Jang, S., et al., *Drug Delivery and Transport to Solid Tumors*. Pharm Res, 2003. **20**(9): p. 1337-1350.
238. Adamski, J., et al., *Hypoxia-induced cytotoxic drug resistance in osteosarcoma is independent of HIF-1Alpha*. PLoS One, 2013. **8**(6): p. e65304.
239. Klein, O.J., et al., *In Vitro Optimization of EtNBS-PDT against Hypoxic Tumor Environments with a Tiered, High-Content, 3D Model Optical Screening Platform*. Mol Pharm, 2012. **9**(11): p. 3171-3182.
240. Chen, Q., et al., *Improvement of Tumor Response by Manipulation of Tumor Oxygenation During Photodynamic Therapy*. Photochem Photobiol, 2002. **76**(2): p. 197-203.
241. Maas, A.L., et al., *Tumor vascular microenvironment determines responsiveness to photodynamic therapy*. Cancer Res, 2012. **72**(8): p. 2079-88.
242. Fuchs, J. and J. Thiele, *The Role of Oxygen in Cutaneous Photodynamic Therapy*. Free Radic Biol Med, 1998. **24**(5): p. 835-847.
243. Filip, A.G., et al., *Photodynamic therapy--indications and limits in malignant tumors treatment*. Rom J Intern Med, 2008. **46**(4): p. 285-93.

244. Metzen, E., et al., *Pericellular PO₂ and O₂ consumption in monolayer cell cultures*. Respir Physiol, 1995. **100**(2): p. 101-106.
245. Jensen, M.D., D.F.H. Wallach, and P. Sherwood, *Diffusion in tissue cultures on gas-permeable and impermeable supports*. J Theor Biol, 1976. **56**(2): p. 443-458.
246. Celli, J.P., et al., *Quantitative imaging reveals heterogeneous growth dynamics and treatment-dependent residual tumor distributions in a three-dimensional ovarian cancer model*. J Biomed Opt, 2010. **15**(5): p. 051603.
247. Wang, W., et al., *3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells*. Biomaterials, 2009. **30**(14): p. 2705-2715.
248. Botchkina, G.I., et al., *New-generation taxoid SB-T-1214 inhibits stem cell-related gene expression in 3D cancer spheroids induced by purified colon tumor-initiating cells*. Mol Cancer, 2010. **9**: p. 192.
249. Korff, T. and H.G. Augustin, *Integration of Endothelial Cells in Multicellular Spheroids Prevents Apoptosis and Induces Differentiation*. J Cell Biol, 1998. **143**(5): p. 1341-52.
250. Xu, W.H., et al., *Doxorubicin-mediated radiosensitivity in multicellular spheroids from a lung cancer cell line is enhanced by composite micelle encapsulation*. Int J Nanomedicine, 2012. **7**: p. 2661-71.
251. Smalley, K.S., M. Lioni, and M. Herlyn, *Life isn't flat: taking cancer biology to the next dimension*. In Vitro Cell Dev Biol Anim, 2006. **42**(8-9): p. 242-7.

252. Subramaniam, D., et al., *Curcumin induces cell death in esophageal cancer cells through modulating Notch signaling*. PloS one, 2012. **7**(2): p. e30590.
253. Glaser, V., *Novel 3D Cell Culture Systems*. 2013.
254. Sergeeva, N.N., et al., *Simple but powerful: Phenanthroline-based small molecules for cellular imaging and cancer screening*. Bioorganic and Medicinal Chem Let, 2011. **21**(15): p. 4385-4388.
255. Faute, M., et al., *Distinctive alterations of invasiveness, drug resistance and cell-cell organization in 3D-cultures of MCF-7, a human breast cancer cell line, and its multidrug resistant variant*. Clin Exp Metastasis, 2002. **19**(2): p. 161-167.
256. Olive, P.L. and R.E. Durand, *Drug and radiation resistance in spheroids: cell contact and kinetics*. Cancer Metastasis Rev, 1994. **13**(2): p. 121-38.
257. Kolchinsky, A. and I.B. Roninson, *Drug resistance conferred by MDR1 expression in spheroids formed by glioblastoma cell lines*. Anticancer Res, 1997. **17**(5a): p. 3321-7.
258. Francia, G., et al., *Gene expression analysis of tumor spheroids reveals a role for suppressed DNA mismatch repair in multicellular resistance to alkylating agents*. Mol Cell Biol, 2004. **24**(15): p. 6837-49.
259. Weissleder, R. and M.J. Pittet, *Imaging in the era of molecular oncology*. Nature, 2008. **452**(7187): p. 580-589.
260. Berezin, M.Y. and S. Achilefu, *Fluorescence Lifetime Measurements and Biological Imaging*. Chem Rev, 2010. **110**(5): p. 2641-2684.
261. Palmer, G.D., et al., *A simple, lanthanide-based method to enhance the transduction efficiency of adenovirus vectors*. Gene Ther, 2008. **15**(5): p. 357-363.

262. Gschneidner, K.A., J.-C. Bünzli, and V.K. Pecharsky, *Handbook on the physics and chemistry of rare earths*. Vol. 34. 2004: Access Online via Elsevier.
263. Parker, D. and J.A. Williams, *Responsive luminescent lanthanide complexes*. *Met Ions Biol Syst*, 2003. **40**: p. 233-280.
264. dos Santos, C.M.G., et al., *Recent developments in the field of supramolecular lanthanide luminescent sensors and self-assemblies*. *Coord Chem Rev*, 2008. **252**(23-24): p. 2512-2527.
265. Tsukube, H., et al., *Dendrimer container for anion-responsive lanthanide complexation and "on-off" switchable near-infrared luminescence*. *Chem Commun*, 2007(24): p. 2533-2535.
266. Pandya, S., J. Yu, and D. Parker, *Engineering emissive europium and terbium complexes for molecular imaging and sensing*. *Dalton Trans*, 2006 (23): p. 2757-2766.
267. Parker, D., et al., *Being excited by lanthanide coordination complexes: Aqua species, chirality, excited-state chemistry, and exchange dynamics*. *Chem Rev*, 2002. **102**(6): p. 1977-2010.
268. Hamacek, J., M. Borkovec, and C. Piguet, *Simple thermodynamics for unravelling sophisticated self-assembly processes*. *Dalton Trans*, 2006. **6**(12): p. 1473-1490.
269. Piguet, C., et al., *Strict self-assembly of polymetallic helicates: The concepts behind the semantics*. *Coord Chem Rev*, 2005. **249**(5-6): p. 705-726.
270. Giuliano, K.A., et al., *High-content screening: A new approach to easing key bottlenecks in the drug discovery process*. *J Biomol Screen*, 1997. **2**(4): p. 249-259.

271. Abraham, V.C., D.L. Taylor, and J.R. Haskins, *High content screening applied to large-scale cell biology*. Trends in Biotechnology, 2004. **22**(1): p. 15-22.
272. Lincoln, P. and B. Nórdén, *Binuclear ruthenium(n) phenanthroline compounds with extreme binding affinity for DNA*. Chem Commun, 1996(18): p. 2145-2146.
273. Sergeeva, N.N., et al., *Simple but powerful: Phenanthroline-based small molecules for cellular imaging and cancer screening*. Bioorg Med Chem Lett, 2011. **21**(15): p. 4385-4388.
274. Bencini, A. and V. Lippolis, *1,10-Phenanthroline: A versatile building block for the construction of ligands for various purposes*. Coord Chem Rev, 2010. **254**(17–18): p. 2096-2180.
275. Cockrell, G.M., et al., *Enhanced Metal Ion Selectivity of 2,9-Di-(pyrid-2-yl)-1,10-phenanthroline and Its Use as a Fluorescent Sensor for Cadmium(II)*. J Am Chem Soc, 2008. **130**(4): p. 1420-1430.
276. Butler, H.M., et al., *BACTERICIDAL ACTION OF SELECTED PHENANTHROLINE CHELATES AND RELATED COMPOUNDS*. Aust J Exp Biol Med, 1969. **47**(5): p. 541-552.
277. McCann, M., et al., *In vitro and in vivo studies into the biological activities of 1,10-phenanthroline, 1,10-phenanthroline-5,6-dione and its copper(ii) and silver(i) complexes*. Toxicol Res, 2012. **1**(1): p. 47-54.
278. Day, T.A. and G.Z. Chen, *The metalloprotease inhibitor 1,10-phenanthroline affects Schistosoma mansoni motor activity, egg laying and viability*. Parasitology, 1998. **116** (Pt 4): p. 319-25.
279. Zieske, J. and G. Bukusoglu, *Effect of protease inhibitors on corneal epithelial migration*. Invest Ophthalmol Vis, 1991. **32**(7): p. 2073-2078.

280. Feder, J., L.R. Garrett, and D. Kochavi, *Studies on the inhibition of neutral proteases by 1,10-phenanthroline*. Biochim Biophys Acta, 1971. **235**(2): p. 370-377.
281. Joshi, H.S., R. Jamshidi, and Y. Tor, *Conjugated 1,10-Phenanthrolines as Tunable Fluorophores*. Angewandte Chemie International Edition, 1999. **38**(18): p. 2721-2725.
282. Puckett, C.A., R.J. Ernst, and J.K. Barton, *Exploring the cellular accumulation of metal complexes*. Dalton Trans, 2010. **39**(5): p. 1159-1170.
283. Rizzuto, R. and T. Pozzan, *Microdomains of intracellular Ca²⁺: Molecular determinants and functional consequences*. Physiol Rev, 2006. **86**(1): p. 369-408.
284. Ferri, K.F. and G. Kroemer, *Organelle-specific initiation of cell death pathways*. Nat Cell Biol, 2001. **3**(11): p. E255-263.
285. De Brito, O.M. and L. Scorrano, *Mitofusin 2 tethers endoplasmic reticulum to mitochondria*. Nature, 2008. **456**(7222): p. 605-610.
286. Beluffi, G., *The impact of tumor biology on cancer treatment and multidisciplinary strategies*. Radiol Med, 2010. **115**(1): p. 172-173.
287. Faulkner, S., S.J.A. Pope, and B.P. Burton-Pye, *Lanthanide complexes for luminescence imaging applications*. Appl Spectrosc Rev, 2005. **40**(1): p. 1-31.
288. Bünzli, J.C.G. and C. Piguet, *Taking advantage of luminescent lanthanide ions*. Chem Soc Rev, 2005. **34**(12): p. 1048-1077.
289. Binnemans, K., *Lanthanide-based luminescent hybrid materials*. Chemical Reviews, 2009. **109**(9): p. 4283-4374.

290. Moore, E.G., A.P.S. Samuel, and K.N. Raymond, *From antenna to assay: lessons learned in lanthanide luminescence*. Acc Chem Res, 2009. **42**(4): p. 542-552.
291. Faulkner, S. and J.L. Matthews, *Fluorescent complexes for biomedical applications*. Compr Coord Chem II, 2004. **9**.
292. Bünzli, J.C.G., *Lanthanide luminescence for biomedical analyses and imaging*. Chem Rev, 2010. **110**(5): p. 2729-2755.
293. Eliseeva, S.V. and J.C.G. Bünzli, *Lanthanide luminescence for functional materials and bio-sciences*. Chemical Society Reviews, 2010. **39**(1): p. 189-227.
294. Montgomery, C.P.M., et al., Acc. Chem. Res., 2009: p. 925-937.
295. Helm, L. and A.E. Merbach, *Inorganic and Bioinorganic Solvent Exchange Mechanisms*. Chem Rev, 2005. **105**(6): p. 1923-1960.
296. Viswanathan, S., et al., *Alternatives to gadolinium-based metal chelates for magnetic resonance imaging*. Chem Rev, 2010. **110**(5): p. 2960-3018.
297. Charbonnière, L.J., et al., *Luminescent lanthanide complexes of a bis-bipyridine-phosphine-oxide ligand as tools for anion detection*. J Am Chem Soc, 2002. **124**(26): p. 7779-7788.
298. Sabbatini, N., et al., *Synthesis and luminescence of lanthanide complexes of a branched macrocyclic ligand containing 2,2'-bipyridine and 9-Methyl-1,10-phenanthroline subunits*. Inorg Chem, 1994. **33**(5): p. 955-959.
299. Senge, M.O. and N.N. Sergeeva, *The chemistry of functional groups*. The Chemistry of Organozinc Compounds, Part 1, Photochemical

Transformations Involving Zinc Porphyrins and Phthalocyanines, 2006:
p. 395-419.

300. Ke, H., et al., *Responsive and mitochondria-specific ruthenium(ii) complex for dual in vitro applications: Two-photon (near-infrared) induced imaging and regioselective cell killing*. Chem Commun, 2010. **46**(36): p. 6678-6680.
301. Kieltyka, R., et al., *Platinum phenanthroimidazole complexes as G-quadruplex DNA selective binders*. Chem - Eur J, 2008. **14**(4): p. 1145-1154.
302. Li, Y.Y., et al., *Cellular internalization and in Vivo tracking of thermosensitive luminescent micelles based on luminescent lanthanide chelate*. ACS Nano, 2008. **2**(1): p. 125-133.
303. Liu, Y., et al., *A Luminescent β -cyclodextrin-based $ru(phen)_3$ complex as dna ompactor, enzyme inhibitor, and translocation tracer*. ACS Nano, 2007. **1**(4): p. 313-318.
304. Liu, F., et al., *The pH-Induced Emission Switching and interesting DNA-Binding Properties of a Novel Dinuclear Ruthenium(II) Complex*. Inorg Chem, 2004. **43**(5): p. 1799-1806.
305. Liu, J., et al., *Synthesis, characterization and antitumor activity of a series of polypyridyl complexes*. Met-Based Drugs, 2000. **7**(6): p. 343-348.
306. Neugebauer, U., et al., *Ruthenium polypyridyl peptide conjugates: Membrane permeable probes for cellular imaging*. Chem Commun, 2008(42): p. 5307-5309.

307. Thomas, S., et al., *Filtering toxicity from the pipeline: The role of high content toxicology in early identification of toxic liabilities*. *Toxicol Lett*, 2011. **205**: p. S177-S178.
308. Helmy, I.M. and A.M. Azim, *Efficacy of ImageJ in the assessment of apoptosis*. *Diagn Pathol*, 2012. **7**: p. 15.
309. Filippi-Chiela, E.C., et al., *Nuclear Morphometric Analysis (NMA): Screening of Senescence, Apoptosis and Nuclear Irregularities*. *PloS one*, 2012. **7**(8): p. e42522.

This work was supported by grants from Science Foundation Ireland (P.I. 09/IN.1/B2650), Fundação da Ciência e Tecnologia (Grant SFRH/BD/62364/2009), and the Health Research Board (HRB Translational Research Award 2007 TRA/2007/11). We are grateful to Dr. Annegret Preuß and Dr. Natalya Seergeva for assistance and to Dr. B. Röder for the illumination set-up.