DEGREE FINAL PROJECT

“Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment”

DFP submitted to obtain the title of DEGREE in BIOMEDICAL ENGINEERING by Olga Ciutad Castejón

Barcelona, June 30th 2015

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The experimental work defined in this project has been carried out in the Institute for Biomedical Imaging and Life Sciences (IBILI) and in the Unity of Biophysics of the Faculty of Medicine of the University of Coimbra.
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ABSTRACT

Nowadays, colorectal cancer is the third most common cancer worldwide. There are some therapies to treat this cancer, such as radiotherapy and chemotherapy. However, their efficacy can be decreased or completely inhibited by one characteristic of the tumor cells, hypoxia. Recently, has appeared one alternative therapy for those patients with inoperable colon cancer and it uses light sources to induce cell death by the production of singlet oxygen and other species of ROS. This therapy is called photodynamic therapy (PDT) but its effectiveness can be affected too by the hypoxic tumor cells. To increase the efficacy of PDT treatment it has been used the hyperbaric oxygen therapy (HBOT), which overcomes the hypoxic state of cancer cells. The combined therapies have been applied using different conditions and their effect has been analyzed by MTT assay and cell cycle and cellular viability analysis by cytometry. The obtained results revealed that combination of PDT and HBOT is promising and that results of combination of PDT followed by HBOT during 60 minutes should be further studied.
RESUMEN

El tercer cáncer más común hoy en día es el cáncer colorrectal. Existen varios tratamientos para eliminarlo, como la radioterapia y quimioterapia. La eficacia de las terapias anticancerígenas puede ser disminuida o anulada por una característica de las células tumorales, la hipoxia. Ha surgido recientemente una terapia alternativa para pacientes con cáncer colorrectal que no puede ser operado y que utiliza fuentes de luz para eliminar los tejidos cancerosos mediante la producción de oxígeno singlete y otras especies de ERO. Esta terapia se llama terapia fotodinámica (TF) pero su efectividad también puede verse afectada por las células tumorales hipóxicas. Para mejorar la eficacia de la TF en el tratamiento de cáncer de colon se ha utilizado la terapia de oxígeno hiperbárico (TOHB), que elimina el estado de hipoxia celular. La combinación de las terapias ha sido aplicada usando diversas condiciones y su efecto se ha analizado mediante los ensayos de MTT y el análisis de ciclo celular y viabilidad celular por citometría. Los resultados obtenidos dan una visión esperanzadora de la combinación de TF con TOHB y en análisis futuros, deberán ser corroborados los resultados de la terapia de TF seguida de TOHB durante 60 minutos.
RESUM

El tercer càncer més comú avui dia és el càncer colorectal. Existeixen diverses teràpies per combatre’l com la radioteràpia i la quimioteràpia. L’eficàcia de les teràpies anticancerígenes es pot veure disminuïda o anul·lada per una característica de les cèl·lules cancerígenes, la hipòxia. Ha sorgit recentment una teràpia alternativa per als pacients amb càncer de colon que no pot ser operat. Aquesta utilitza fonts de llum per eliminar el teixit cancerós mitjançant la producció d’oxigen singlet i altres espècies de ERO, s’anomena teràpia fotodinàmica (TF). No obstant, la seva efectivitat també es pot veure afectada per les cèl·lules tumorals hipòxiques. Per millorar la seva eficàcia en el tractament del càncer colorectal s’ha utilitzat la teràpia d’oxigen hiperbàric (TOHB), que elimina l’estat d’hipòxia cel·lular. La combinació de les teràpies ha sigut aplicada utilitzant diverses condicions i el seu efecte s’ha analitzat mitjançant l’assaig MTT i l’anàlisi de cicle cel·lular i de viabilitat cel·lular per citometria. Els resultats obtinguts donen una visió esperançadora de la combinació de TF i TOHB i en anàlisis futurs, s’haurà de corroborar els resultats de la teràpia de TF seguida de TOHB durant 60 minuts.
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The main purpose of the present project is to evaluate the combination of hyperbaric oxygen therapy (HBOT) and photodynamic therapy (PDT) in colorectal adenocarcinoma cell line as a new alternative therapy to treat this cancer. *In vitro* studies have been performed to verify the therapeutic outcome produced by the combination of both therapies in different conditions.

Cells were incubated with the photosensitizer (PS) 5,15-bis(2-bromo-3-hydroxyphenyl) chlorin (BBr2HPC) to perform PDT by irradiation. To create conditions of increased oxygen partial pressure a prototype has been used in order to summit the cells to HBOT conditions. After summiting the cells to corresponding therapies, the effect on metabolic activity (MTT), viability and types of cell death (annexin V and propidium iodine double labeling by flow cytometry) and the alterations in the cell cycle (propidium iodine by flow cytometry) were evaluated.

Therapies applied consisted in perform PDT based on BBr2HPC by incubating cells with different concentrations of PS and by irradiating with a total energy of 10J. HBOT can be applied immediately before or after PDT, by a period of 30min or 60min. Evaluation of metabolic activity is performed both 24h and 48h after therapies. To evaluate cell viability and cell cycle alterations we studied the therapy performed with the EC$_{50}$ value previously determined to BBr2HPC (Laranjo 2014) combined with HBOT during 60min, after PDT.
CHAPTER 2: INTRODUCTION

The aim of this chapter is to expose all the theoretical foundations needed for the complete understanding of the present project. In the course of this chapter it is going to be explained the kind of cancer studied, its main cell characteristic and the therapies applied to treat this cancer cell line.

2.1 Colorectal adenocarcinoma

Cancer is one of the main mortality causes all over the world with 8,2 millions of deaths and 14 millions of new diagnosis in 2012. (World Health Organization, WHO; U.S National Library of Medicine) Cancer cell alters its metabolism in response to a challenging environment by promoting cell growth and proliferation, diverging significantly from normal tissues. (Abrantes et al. 2014) Increased rate of multiplication of abnormal cells makes possible their extension to different parts of the body, this process is known as metastasis. Metastasis is the main cause of dead by cancer. One of the cancers more diagnosed between men and women is colorectal cancer. (WHO; U.S National Library of Medicine)

Colorectal cancer is formed in the tissues of the colon or in the tissues of the rectum, both in the large intestine. Most colorectal cancers are adenocarcinomas, cancers that begin in cells that make and release mucus and other fluids. (National Cancer Institute, NCI)
General information about colorectal cancer can be found in the next sections.

### 2.1.1 Epidemiology

Worldwide, colorectal cancer is the third most common form of cancer. (International Agency for Research on Cancer, IARC) Colorectal cancer occurs when tumors form in the lining of the large intestine formed by colon, rectum and anus as it is shown in **Figure 1**. Colorectal cancer usually develops from a polyp, a benign tumor found in the walls of the colon or rectum. Its development is slow and it can take few years to turn into cancer if undetected or ignored. The type of polyp with biggest risk to become cancerous is adenoma. (American Society for Gastrointestinal Endoscopy, ASGE; U.S National Library of Medicine)

The risk of developing colon cancer can depend on hereditary factors, which are the high-risk group, and the non-hereditary factors, that can reduce its probability by having a healthy lifestyle. Limiting screening or early cancer detection to only high-risk groups would miss the majority of colorectal cancers. (NCI; U.S National Library of Medicine) The factors that increase the risk of rectal cancer include the following: (ASGE; Burkitt 1993; NCI; U.S National Library of Medicine)

- **Hereditary factors**: hereditary nonpolyposis colorectal cancer, hereditary polyposis disorders, hamartomatous disorders, personal or first-degree relative (parent, sibling or offspring) history of colorectal cancer or colorectal adenomas and personal history of ovarian, endometrial or breast cancer.
- **Non-Hereditary factors**: upper age than 50, a diet high in fat and obesity, smoke and medium or high consumption of alcohol and sedentary lifestyle.

It is a highly treatable and often curable disease when localized to the bowel. Surgery is the primary form of treatment and results in cure in approximately 50% of the patients. Recurrence following surgery is a major problem and is often the ultimate cause of death. (U.S National Library of Medicine)

About 54% of colorectal cancer cases occurred in the most developed countries, being the highest incidence of colorectal cancer in Oceania and Europe and the lowest incidence in Africa and Asia. In **Figure 2** it is shown the estimated incidence and mortality worldwide for the year 2012. (IARC)

**Figure 2.** Estimated incidence and mortality worldwide of colon cancer in 2012. (adapted from IARC)
2.1.2 Classification, clinical presentation and diagnosis

The American Joint Committee on Cancer (AJCC) has designated the TNM \(^1\) classification to define colorectal cancer. It consists in 4 stages defined by the growth of the primary tumor (T), the propagation to the regional lymph nodes (N) and the propagation to other organs or distant metastasis (M).

Stage 1 colorectal adenocarcinomas are small and confined to the colon and stage 4 tumors have spread beyond areas near the colon and other parts of the body. Stages between 2 and 3 describe conditions in between these two extremes. In Figure 3 is represented this progression.

Colon adenocarcinoma progresses slowly and may not present symptoms for up to five years. As the cancer grows, symptoms become more likely and can include rectal bleeding, fatigue, shortness of breath, angina and changes in bowel habits, abdominal discomfort and anemia or bowel obstruction. Diarrhea, constipation, weight loss with no known reason, nausea and vomiting are also symptoms described. (NCI)

\(^1\) The TNM classification can be found in the National Cancer Institute website, which is the following: http://www.cancer.gov/cancertopics/pdq/treatment/rectal/HealthProfessional/page4/page3.
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Figure 3. Stage of colorectal cancer progression. (a) Stage I: the cancer is found in the mucosa, submucosa and muscle layers. (b) Stage II: it spreads through the serosa and can reach nearby organs. (c) Stage III: cancer reaches the lymph nodes and nearby organs. (d) Stage IV: cancer spreads through the blood and lymph nodes to other parts of the body. (adapted from NCI)

Because most colon adenocarcinomas do not present symptoms it is important to have screening tests, especially those population over 50. About 5 to 10 percent of colon cancers are initially discovered during a digital rectal exam, where abnormal areas are searched. Also, the presence of blood in the stool can be a sign for cancer or polyp. (American Cancer Society, ACS; NCI)

The tests and examinations used for colorectal cancer detection include:

- **Blood test**: it can be a complete blood count to find possible anemia or it can check liver enzymes for a possible propagation of the cancer to the liver and tumor markers.
- **Colonoscopy**: endoscopic examination of the entire colon and rectum. It provides a visual diagnosis and special instruments can be passed through the colonoscope to biopsy or remove any suspicious-looking areas such as polyps, if needed.
- **Sigmoidoscopy**: is like a colonoscopy but just examining the last part of the colon.
- **Double-contrast barium enema**: X-ray scan of the colon and rectum.
In some situations imaging techniques may be ordered to check if the tumor has spread to other organs or parts of the body and especially to the lungs, lymph nodes, liver or ovaries. Those include: computed tomography (CT), ultrasonography (US), magnetic resonance imaging (MRI), X-ray, angiography and positron emission tomography (PET) scan. (ACS and NCI)

2.1.3 Treatment

Depending on the stage of the cancer, two or more types of treatment may be combined at the same time or used before or after another. The main types of treatment that can be used for colon and rectal cancer are:

- **Surgery**: can usually cure it when it is found early. Is generally recommended for 90 percent of patients.

- **Radiation therapy**: can be used to shrink tumors or to destroy cancer cells that remain after surgery.

- **Chemotherapy**: recommended if the cancer has spread. Can be used before and after surgery and can be combined with immunotherapy or radiation therapy.

- **Ablation or embolization**: destroy tumors without removing them or try to block or reduce the blood flow to cancer cells by injecting substances. Used for advanced cancer or when it has spread to the liver or other parts.

Other factors to consider as well as the stage of the cancer include the overall health, the likely side effects of the treatment, and the probability of curing the disease, extending life or relieving symptoms. (ACS; NCI)

**Photodynamic therapy** (PDT) is a relatively new alternative modality for patients with colorectal cancers unsuitable for operation. The important biological advantages of PDT are that the risk of perforation is small even if full thickness necrosis is produced to eradicate the tumor and the areas of necrosis heal predominantly by regeneration with little scarring. The major disadvantage of PDT is that the amount of tumor destruction is limited by the penetration of light. (Barr et al. 1990)
2.2 Hypoxia in cancer cells

Hypoxia is a common characteristic of tumoral cells, becoming a key factor for tumor progression and resistance to anticancer therapy. The hypoxic tissue areas have O$_2$ tensions (pO$_2$ values) lower than 2,5 mmHg. Hypoxic areas arise as a result of an imbalance between the supply and consumption of oxygen, being the result of an imbalance between tissue growth and the development of new vasculature. Areas with a poorer oxygenation than their respective normal tissues have been found in cancers of the vulva, prostate, rectum, pancreas, lung, brain tumors, soft tissue sarcomas, non-Hodgkin’s lymphomas, malignant melanomas, metastatic liver tumors, renal cell cancer and breast, uterine cervix and head and neck cancer. Hypoxic areas are also independent of clinical size, stage, histology, grade, nodal status or patient demographics. Local tumor recurrences have a higher hypoxic fraction than the respective primary tumors. (Abrantes et al. 2014; Daruwalla and Christophi 2006; Vaupel and Mayer 2007)

The growth of tumors is limited by the delivery of oxygen, nutrients and the removal of waste products. As a tumor grows, cells undergo nutrient deprivation and acidosis and they become hypoxic making its microenvironment toxic. Tumor cells can adapt to the ischemic and low nutrient microenvironment by three main adaptations: forming an aberrant vascular network, evading apoptotic destruction and switching to anaerobic glycolysis. All three mechanisms are driven by the hypoxic tumor adverse microenvironment and make tumor cells survive. (Abrantes et al. 2014; Daruwalla and Christophi 2006)

Hypoxia regulates many pathways including angiogenesis, glycolysis, metastasis, apoptosis and pH regulation, among others, by affecting the expression of many gene products that are involved in the pathways mentioned. (Daruwalla and Christophi 2006)

Hypoxia has been suggested as an adverse prognostic factor for patient outcome. There is an unfavorable therapeutic response and a worse disease-free survival for patients with hypoxic cancers or soft tissue sarcomas. Regions of hypoxia of tumors are associated with slowly proliferating cells. Those cells are mostly resistant to chemotherapy due to rapidly dividing cells targets of the standard chemotherapy but it has also been shown to diminish the efficacy of certain regimens of radiotherapy, photodynamic therapy and immunotherapy.
The degree of inhibition depends on the severity and duration of hypoxia. (Sudhakar et al. 2013; Vaupel and Mayer 2007)

2.3 Hyperbaric oxygen therapy (HBOT)

One unique feature of tumors is the presence of hypoxic regions, which occur predominantly at the tumor inner part. Hypoxia has a major impact on various aspects of tumor cell function and proliferation. Hypoxic tumor cells are relatively insensitive to conventional therapy due to cellular adaptations caused by the hypoxic microenvironment. To alter the hypoxic state and to reverse these adaptations and improve treatment outcome, one way to increase tumor oxygen tensions is by hyperbaric oxygen therapy (HBOT). (Daruwalla and Christophi 2006; Ogawa et al. 2013)

2.3.1 What is it?

HBOT is defined by the Undersea and Hyperbaric Medical Society (UHMS) as a treatment in which a patient intermittently breathes 100% oxygen while the treatment chamber is pressurized to a pressure greater than sea level (1atm). The pressure increase must be systemic and may be applied in monoplace (single person) or multiplace chambers.

Physiologically, short-term effects of hyperbaric oxygen (HBO) include vasoconstriction compensated by increased plasma oxygen carriage, enhanced oxygen delivery to ischemic tissues, reduction of edema, phagocytosis activation and an anti-inflammatory effect, enabling normal host responses to infection and ischaemia. Long-term effects of HBO include neovascularization, osteogenesis and the stimulation of collagen formation by fibroblasts. (Attrick et al. 1996; Daruwalla and Christophi 2006; Gill and Bell 2004; Ogawa et al. 2013)

Hyperoxia as a result of HBOT also induce the formation of reactive oxygen species (ROS) or free radicals, which can damage tumors by inducing excessive oxidative stress. In nonmalignant cells, ROS levels are relatively low and regulated and play a duel role in tumor growth. Initially, at low levels, ROS aid tumor progression via DNA damage and uncontrolled proliferation of a genomically unstable and highly aggressive cell line. In excess however, ROS become toxic to tumor cells inducing to a programmed cellular death, named
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apoptosis. The most damaging active oxygen species are superoxide anion (O$_2^-$), hydroxyl radical (OH·), hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (¹O$_2$).

The effect of HBOT is dependent on the tumor’s type and stage and the HBOT regimen, timing, duration, atmospheric pressure and number of HBO exposures. Although most of the experimental and clinical studies suggest that HBOT has no direct effect on tumors growth and remains ineffective as a stand-alone therapy, may enhance the efficacy of certain therapies that are limited due to the hypoxic tumor microenvironment when used in an adjuvant setting with certain types of malignancy. (Daruwalla and Christophi 2006)

Some treatments used in combination with HBOT to treat malignant tumors are radiotherapy, chemotherapy and photodynamic therapy. The use of HBOT as an adjuvant treatment is justified by the following: (Daruwalla and Christophi 2006; Ogawa et al. 2013)

- Improved oxygenation improves drug delivery to hypoxic regions in the tumor.
- Remove the hypoxic stimulus that drives angiogenesis and may also cause cells to enter a proliferative stage, thus sensitizing them to others therapies.
- Increasing intratumoral ROS levels beyond the threshold may induce tumor destruction.

2.3.2 Therapeutic uses

In general, high-grade gliomas, advanced head and neck cancers and advanced uterine cervical cancers in particular have large numbers of hypoxic cells that exhibit poor responses to therapy. (Ogawa et al. 2013) Clinically, HBO has been investigated as an adjuvant therapy when combined with radiotherapy, chemotherapy and photodynamic therapy to treat malignant tumors: (Daruwalla and Christophi 2006)

- **Radiotherapy** induces DNA damage through the ionization of oxygen to produce ROS. Several studies have reported that radiotherapy immediately after HBOT was safe and seemed to be effective in patients
with high-grade gliomas. Moreover, the addition of HBO may protect normal tissues from radiation injury. (Ogawa et al. 2013)

- HBO may help overcome chemotherapy resistance in hypoxic tumors by increasing tumor perfusion and cellular sensitivity. HBOT in combination with chemotherapy increases cellular uptake of certain anticancer drugs and the susceptibility of cells to these drugs.

- The response to photodynamic therapy depends on adequate tumor oxygenation as well as sufficient intratumoral accumulation of the photosensitizing agent so HBO may improve the effects of PDT by improving tumor perfusion and increasing ROS production, specifically the amount of singlet oxygen.

In hypoxic conditions, HBO reduces infection and cell-death and maintains tissue viability while healing occurs. HBOT is widely accepted as the only treatment for the conditions found on the UHMS lists in Table 1 for which research data and extensive positive clinical experience, with a varying evidence base, have become recommended and used for a wide range of medical conditions. (Atrick et al. 1996 and Gill and Bell 2004)

**Table 1.** UHMS approved indications for hyperbaric oxygen therapy, diseases for which HBOT is currently used. (adapted from Atrick et al. 1996, Gill and Bell 2004; Ogawa et al. 2013)

- Arterial gas embolism,
- Carbon monoxide poisoning; cyanide poisoning; smoke inhalation,
- Clostridial myostitis and myonecrosis (gas gangrene),
- Crush injuries, compartment syndromes and other acute traumatic ischemic injury,
- Decompression sickness (DCS),
- Problem wounds,
- Anemia due to exceptional blood loss,
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- Intracranial abscess,
- Necrotizing soft tissue infections (necrotizing fasciitis),
- Refractory osteomyelitis,
- Compromised skin grafts and flap,
- Delayed radiation-induced tissue injury (LRTI),
- Thermal burns.

HBOT has been proposed for other conditions, shown in Table 2, that have been not approved by the UHMS yet.

**Table 2. Other suggested indications for HBOT. (adapted from Gill and Bell 2004)**

<table>
<thead>
<tr>
<th>Acute cerebrovascular incidents,</th>
<th>Spinal cord injury,</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral oedema,</td>
<td>Intra-abdominal abscess,</td>
</tr>
<tr>
<td>Head injury,</td>
<td>Acute central retinal artery insufficiency,</td>
</tr>
<tr>
<td>Meningitis,</td>
<td>Brown recluse spider bite,</td>
</tr>
<tr>
<td>Ischaemia-reperfusion injury,</td>
<td>Sickle cell crisis,</td>
</tr>
<tr>
<td>Lepromatous leprosy,</td>
<td>Fracture healing and bone grafting,</td>
</tr>
<tr>
<td>Pseudomonas colitis,</td>
<td>Hydrogen sulphate or carbon tetrachloride poisoning.</td>
</tr>
</tbody>
</table>

**2.3.3 How is it used?**

To be effective, HBO must be inhaled in the atmosphere or administrated through an endotracheal tube in monoplace chambers or through masks, tight-fitting hoods or endotracheal tubes in multiplace chambers. Their portability,
minimal personnel requirements and relatively low cost have made monoplace chambers the most common type of chamber worldwide. (Atrick et al. 1996; Gill and Bell 2004) It can also be found mobile multiplace chambers and portable monoplace chambers. That equipment is shown in **Figure 4**.

![Types of hyperbaric chambers](image)

**Figure 4.** Types of hyperbaric chambers (a) Standard monoplace chamber (Sigma36, Perry Baromedical) (b) Portable monoplace chamber (Solace210, OxyHealth) (c) Standard multiplace chamber (HAUX-STARMED 2200, HAUX Life Support) (d) Mobile multiplace chamber (OxyHeal 4000-T, OxyHeal Health Group).

The duration of single treatments varies from 45 minutes for carbon monoxide poisoning to several hours for some severe decompression disorders and for treatment of wounds that do not respond to debridement or antibiotics most
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protocols average 90 minutes for each of 20 to 30 treatments. (Atrick et al. 1996)

HBO when applied as an adjuvant therapy can be administered simultaneously, previous to irradiation to increase the oxygen tension of hypoxic tumor cells or after irradiation to reduce radiation-induced tissue injury. However, the administration of HBO inside a pressure chamber while patients are irradiated is difficult and costly; a limited number of hyperbaric facilities are located in the proximity of radiation oncology departments. (Daruwalla and Christophi 2006)

2.3.4 Adverse effects and contraindications

HBO treatment is a relatively safe treatment but carries some risks due to the increased pressure and hyperoxia. According to standard protocols, with oxygen pressures not exceeding 3atm and treatment sessions limited to a maximum of 120 minutes HBOT is safe, however, some adverse effects may occur.

The most common side effect of oxygen toxicity is a progressive and reversible myopia, thought to be due to physical lens deformation consequence of the direct toxic effect. A few patients may experience pain from different barotraumas as a result of rapid pressure changes. The most common are middle ear and cranial sinuses barotraumas but others extremely rare can occur like pulmonary, pneumothorax, inner ear or dental barotraumas.

Psychological side effects such as claustrophobia are common and can be a problem in monoplace chambers. Accidents are a risk due to the enriched oxygen and inaccessibility. (Atrick et al. 1996; Gill and Bell 2004)

The only absolute contraindication to HBO is an untreated tension pneumothorax and this must be excluded before treatment. Relative contraindications include impaired pressure equalization and cardiac disease. (Gill and Bell 2004)

2.3.5 Future perspectives

The discovery of beneficial cellular and biochemical effects has strengthened the rationale for administering hyperbaric oxygen as therapy in patients with some of the diseases mentioned in Table 1. (Atrick et al. 1996)
To date, experimental and clinical evidences of the HBOT effect combined with therapies are limited. The lack of effect of HBO in experimental models as a stand-alone therapy may explain why it has not been investigated extensively in a clinical setting. Nevertheless, by altering oxygen levels in vivo, HBO can improve the radiosensitivity of tumors, enhance photodynamic therapy or enhance oxidative stress and tumor cell kill of certain chemotherapy. The limitations of HBOT include the placing of patients in HBO chambers while simultaneously administered with a cancer therapy. (Daruwalla and Christophi 2006)

HBOT is expensive, not universally available and not without risks; further research is needed to establish its efficacy and safety in other conditions. Consideration should be given as to the cost involved in such combined therapy against the extent of benefit that can be achieved. (Daruwalla and Christophi 2006; Gill and Bell 2004)

2.4 Photodynamic therapy (PDT)

PDT is increasingly being recognized as an alternative treatment modality for solid cancers, like carcinomas, and is able to induce cell death by oxidative stress trough activation with light of a non-toxic photosensitizer. (Teixo 2013; Triesscheijn et al. 2006)

2.4.1 What is it?

PDT involves two relatively simple procedures: the administration of a photosensitizer (PS) followed by local illumination of the tumor with light of the appropriate wavelength to activate the specific drug. Light can then be targeted to the tumor site. Photochemical activation of the photosensitizing agent generates highly toxic singlet oxygen and other ROS, which can cause intracellular death. (Daruwalla and Christophi 2006; Triesscheijn et al. 2006)

Activation of the photosensitizer upon absorption of the light transforms the PS from its ground state (\(^1\)PS) into an excited singlet state (\(^1\)PS\(^*\)). From this state PS may decay directly back to ground state by emitting fluorescence, used clinically for photodetection. However, to obtain a therapeutic photodynamic effect, the photosensitizer must undergo electron spin conversion to its triplet
state ($^{3}\text{PS}^{*}$). This process is shown in Figure 5. Two possible reactions can occur: (Triesscheijn et al. 2006)

- **Type I**: In the presence of oxygen, the excited molecule can react directly with a substrate, by proton or electron transfer, to form radicals or radical ions, which can interact with oxygen to produce oxygenated products.
- **Type II**: The energy of the excited photosensitizer can be directly transferred to oxygen to form singlet oxygen, which is the most damaging species generated during PDT.

**Figure 5.** Principle of PDT. After light activation, the PS transforms into its excited triple state, enabling two possible reactions: type I form radicals and type II singlet oxygen. (Triesscheijn et al. 2006)

Singlet oxygen generated by the photochemical reaction can directly induce tumor cell deaths by the induction of apoptosis and necrosis. In addition to directly elimination of cancer cells, PDT appears to shrink or destroy tumors in two other ways: it can damage the blood vessels of the tumor and surrounding healthy vessels, resulting in indirect tumor kill via the induction of hypoxia and starvation, and also is able to initiate an immune response against the remaining tumor cells. (NCI; Triesscheijn et al. 2006)

There are tumor tissue properties that made tumor cells internalize more PS than the normal tissue such as their lower pH and a higher expression of low density lipoproteins receptors. Together with a local illumination of the tumor by the light source make PDT a high selective therapy able to reduce the risks of damaging normal cells with other therapies like radio and chemotherapy. (Teixo 2013)
The efficacy of PDT in the treatment of cancer depends on the type of photosensitizer, drug concentration and intracellular localization, light dose (fluence), dose rate (fluence rate) and oxygen availability. (NCI; Triesscheijn et al. 2006)

2.4.2 Therapeutic uses

To date, exist a few approved PS for treat some kind of cancers. Those cancers are: bladder cancer, skin cancer, head and neck cancer, esophageal cancer, Barrett’s esophagus and endobronchial cancer. Efficacy is high for small superficial tumors.

This therapy is mainly used in dermatology to treat acne, rosacea, pustules, cutaneous tuberculosis and also it is used in fotorejuvenation. PDT is used in other medical areas like gastroenterology to treat pathologies of the gastrointestinal tract, neurology as coadjutant therapy of tumor surgery and in ophthalmology to treat neovascular membranes between others. (Arias et al. 2007; NCI; Triesscheijn et al. 2006)

2.4.3 How is it used?

Cancer treatment based in PDT is initiated by injecting a photosensitizing agent into the bloodstream. PS is internalized by cells all over the body but its uptake and clearance time is higher in cancer cells than in normal cells. Exposure of the tumor to light is usually made approximately 24 to 72 hours after injection to allowing most of PS to left normal cells but remain in cancer cells. The PS in the tumor absorbs the light and produces an active form of oxygen that destroys nearby cancer cells.

Each PS is activated by light of a specific wavelength and used to treat different areas of the body. This wavelength determines how far the light can travel into the body. The most ideal PS would be a chemically pure drug with preferential uptake in tumor, rapid clearance and a strong absorption peak at wavelengths greater than 630nm, corresponding to the red edge or infrared which is the most penetrating light. (Teixo 2013; Triesscheijn et al. 2006)

Several photosensitizing agents are currently approved by the US Food and Drug Administration (FDA) to treat certain cancers, pre-cancers and other non-
Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment

cancerous diseases. The use of one or another PS depends on the disease to treat and its localization. Those agents approved for clinical practice are shown in Table 3.

**Table 3. PS approved for clinical practice. (adapted from Allison and Sibata 2010; Teixo 2013)**

<table>
<thead>
<tr>
<th>PS group</th>
<th>Commercial name</th>
<th>Chemical name</th>
<th>Clinical uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porphyrin</td>
<td>Photofrin®</td>
<td>HpD</td>
<td>Head and neck and brain tumors (HpD).</td>
</tr>
<tr>
<td></td>
<td>Photogem®</td>
<td>HpD</td>
<td>Skin malignancies, actinic keratosis and superficial basal cell lesions, early and superficial non melanoma cutaneous malignancies, head and neck tumors, Barrett’s esophagus, bladder tumors and prostate cancer (ALA).</td>
</tr>
<tr>
<td></td>
<td>Levulan®</td>
<td>ALA</td>
<td>Macular degeneration, ophthalmic astrocytoma, choroidal melanoma and various cutaneous malignancies (Veterporfin).</td>
</tr>
<tr>
<td></td>
<td>Metvix®</td>
<td>M-ALA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexvix®</td>
<td>H-ALA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Visudine®</td>
<td>Verteporfín</td>
<td></td>
</tr>
<tr>
<td>Texaphyrin</td>
<td>Lu-Tex; Antrin®</td>
<td>Lutexaphyrin</td>
<td>Lung cancer metastasis and cutaneous metastasis for breast cancer.</td>
</tr>
<tr>
<td>Chlorin</td>
<td>Foscan®; LS11;</td>
<td>Temoporfin</td>
<td>Head and neck cancer, tumors of lip and oral cavity and esophageal cancer (Temoporfin).</td>
</tr>
<tr>
<td></td>
<td>Photolon®;</td>
<td>Talaporfin</td>
<td>Recurrent tumors, refractory</td>
</tr>
<tr>
<td></td>
<td>LtxTM;</td>
<td>HPPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ApoptesinTM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The light used for PDT can come from a laser or other sources like lamps and LEDs. Laser light can be directed through fiber optic cables to deliver light to areas inside the body. Light delivery for treatment of large surface areas such as treatment of skin diseases may also be effectively accomplished using fluorescent lamps, and for difficult anatomic areas with curvatures LED can be arranged in different geometric combination besides having a wide emission wavelength range and good power output. (Brancaleon and Moseley 2002; Mang 2004)

The main limitation of PDT is that the light needed to activate most PS cannot pass through more than about 1cm of tissue. For this reason, PDT is usually used to treat tumors on or just under the skin or on the lining of internal organs or cavities. PDT is also less effective in treating large tumors because the light cannot pass far into these tumors. PDT is a local treatment and generally cannot be used to treat cancer that has metastasized.

PDT may also be repeated and may be used with other therapies, such as surgery, radiation, chemotherapy or HBOT. (Teixo 2013; Triesscheijn et al. 2006)
2.4.4 Adverse effects and contraindications

Some of the photosensitizers used in this therapy can make the skin and eyes sensitive to light for approximately 6 weeks after treatment. Thus, patients are advised to avoid direct sunlight and bright indoor light for at least 6 weeks. PS tends to build up in tumors and the activating light is focused on the tumor. As a result, damage to healthy tissue is minimal. However, PDT can cause burns, swelling, pain and scarring in nearby healthy tissue. Other side effects of PDT are related to the area that is treated and they can include coughing, trouble swallowing, stomach pain, painful breathing or shortness of breath; which are usually temporary.

There are no long-term side effects if appropriate protocols are followed. (NCI; Triesscheijn et al. 2006)

The main contraindication to PDT is porphyria, inherited or acquired disorders of certain enzymes, which can be caused by the persistence of some PS. Another contraindication is the coexistence of hepatic diseases or instable cardiac diseases. (Arias et al. 2007)

2.4.5 Future perspectives

Clinical trials and research studies are under way to evaluate the use of PDT for cancers of the brain, skin, prostate, cervix, intestines, stomach and liver. PDT is currently offered in only a few selected centers, although it is slowly gaining acceptance as an alternative to conventional cancer therapies. Other research is focused on the development of PSs that are more powerful, more specifically target cancer cells and are activated by light that can penetrate tissue and treat deep or large tumors as well on ways to improve equipment and the delivery of the activating light. (NCI; Triesscheijn et al. 2006)

PDT is a noninvasive therapy that is consolidated to treat different cancers and other indications in diverse medical areas. The cost of the PSs is the first limit to the use of this treatment. Also, studies about the long term efficacy are required. (Arias et al. 2007)

The combination of PDT with HBOT may enhance the efficacy of PDT improving the PS delivery. Getting a better efficacy allows the reduction of the quantity of
PS used in therapy and directly, can reduce the costs for this treatment and its secondary effects.

Previous work developed by IBILI researchers with the PS BBr2HPC, evaluating its photodynamic action and cytotoxicity in the treatment of colorectal cancer, shown a good performance of the PS in PDT and had promising results in colon cancer treatment by demonstrating an increasing inhibition directly proportional to increasing PS concentration. Those cells were not able to recover, the damage was irreversible. (Laranjo 2014; Teixo 2013)
CHAPTER 3: MATERIALS AND METHODS

In this section are defined the biological samples, chemicals, laboratory equipment and protocols used to see the outcome of the combination of HBOT and PDT and evaluate its effect with the MTT assay and the analysis of cell viability and cell cycle. As well, the software used to process the results of this alternative treatment is specified and complemented with the information provided in Chapter 1: Software that can be found in the Annexes of this project.

3.1 Cells and culture conditions

In this project, it has been studied a human cell line of colorectal adenocarcinoma, WiDr, obtained from the American Type Culture Collection (ATCC, CCL-218). The cell line was thawed and expanded in adherent culture according to supplier recommendations. For all the studies, cells were kept at 37°C in a humidified atmosphere with 95% air and 5% CO₂ in an incubator (Binder C-50). Cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM, Sigma D-5648) supplemented with 5% fetal bovine serum (Sigma F7524), 250μL of sodium pyruvate (Gibco 11360) and 1% of antibiotic (10.000units penicillin, 10mg streptomycin and 25μg amphotericin B per mL; Sigma A5955).
3.2 Combined therapy

The combination of therapies has been studied in two ways, PDT after and before HBOT. The protocol used for each treatment is explained in 3.2.1 and 3.2.2. Culture plates of 24 wells have been used with 100,000 cells/mL. All the plates have no treated control cells and cultured cells with solvent, and different concentrations of PS, in order to observe the effects induced by the PS in cell metabolic activity. Thus, 1 mg/mL concentration of the PS BBr2HPC is dissolved in a mixture of water (H₂O), polyethylene glycol 400 (PEG400) and ethanol (EtOH) (50:30:20, v/v/v). The culture medium is changed before applying PDT to analyze only the effects induced by internalized PS.

3.2.1 Hyperbaric oxygen therapy (HBOT)

Cells are exposed to 100% oxygen (O₂) pressurized to 1 bar in an adapted hyperbaric chamber for the treatment of in vitro cells (Figure 6 (a)). The most wanted characteristic of the chamber is being able to maintain the pressure stable in the pan and it has been possible adjusting a pressure cooker (Silampos Lagos²) in two aspects: the tube of the oxygen tank was directly fitted with the adjustment valve of the pressure cooker and the other security valve was sealed. The pressure cooker has a maximum security pressure of 2.5 bar, which allow us to perform our studies in perfect security conditions. The lid of the pressure cooker has a system that allow to maintain pressure constant and without loss. A decompressing valve was added.

It has been established two different durations for the therapy; 30 min and 60 min. During the treatment the chamber was placed in an incubator at 37°C. This protocol has been improved based on consulted bibliography (Hjelde et al. 2005; Chen et al. 2007; Bosco et al. 2013). The pressure of O₂ is slowly raised until 1 bar to compress the chamber and when the therapy is finished it must be decompressed using a proper valve. HBOT is performed 24 h after incubation of PS, being performed before or after irradiation.

² More characteristics about the pressure cooker Lagos from Silampos can be found on its website, which is the following: http://www.silampos.pt/fotos/editor2/manual_instrucoes_lagos.pdf.
3.2.2 Photodynamic therapy (PDT)

The used photosensitizing agent is 5,15-bis(2-bromo-3-hidroxifenil)chlorin (BBr2HPC). Its maximum absorption is 627nm and it has been developed by IBILI researchers (Laranjo 2013; Serra, et al. 2010). A fluorescent light with a red filter (λcut-off<560nm) has been used as a source of irradiation (Figure 6 (b)). Primarily, cells have been incubated with several concentrations of PS: 5, 15, 25, 50, 100, 250 and 500nM. Irradiation is performed 24h after PS incubation by irradiating cells with a fluence of 7,5mW/cm², until the delivery of a total energy of 10J.

![Figure 6. Equipment used for combined therapy. (a) Hyperbaric chamber used during the laboratory experience placed inside the incubator (b) PDT light source, fluorescent with red filter. (own source)](own source)

3.3 Metabolic activity evaluation (MTT)

Assays that measure metabolic activity are suitable for analyzing indirectly proliferation, viability and cytotoxicity and they are commonly used to monitor the response and viability of cells in culture after treatment with different stimuli. The proper choice of an assay depends on the number and type of cells used as well as the expected outcome.

The MTT assay consists on the reduction of tetrazolium salts by metabolically active cells. Actively proliferating cells increase their metabolic activity while cells exposed to toxins will have decreased activity. It is a colorimetric assay for measure cell metabolic activity and indirectly can be used to assess cell viability.
The assay is performed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (thiazolyl blue or MTT), a water soluble salt when prepared in media or salt solutions acquires a yellowish color.

Active mitochondrial dehydrogenases enzymes of living cells convert the dissolved MTT to an insoluble purple formazan by reducing the tetrazolium ring. Non metabolic active cells do not cause this conversion.

For being able to measure the absorbance it is necessary to dissolve the crystals formed with a solution of HCl and isopropyl alcohol (2-propanol). (Dojindo; Teixo 2013) In order to have the percentage of cellular proliferation, it is measured the absorbance of each cell media and its value is compared with a control solution by spectrophotometry.

To perform this assay cells where previously treated with PDT and HBOT as described in 3.2 Combined therapy. This assay was performed 24h and 48h after the therapy. In total, 8 different conditions were subjected: PDT+HBOT 30min, PDT+HBOT 60min, HBO 30min+PDT and HBO 60min+PDT; after 24h and 48h.

Protocol

To evaluate metabolic activity, the culture medium is discarded and each well of the culture plate is washed with 500μL of phosphate buffer saline (PBS), pH 7,4. After PBS is discarded and 200μL of MTT solution (0,5mg/mL; Sigma, EUA) are added in each well, it is incubated for 2h in an incubator at 37°C in the dark, as MTT is sensible to light. After the incubation, crystals of formazan (Figure 7) are dissolved with 200μL of a 0,04M solution of hydrochloric acid in isopropanol in constant agitation for 30min. When crystals are dissolved, content of each well is transferred to a 96 wells plate in order to measure its absorbance with a spectrometer in the wave length of 570nm and 620nm. The program Gen5 1.09 allows the measurement and display of the absorbance made with the spectrometer, which is connected to the computer.
3.4 Flow cytometry

The flow cytometry is a laser-based technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. This technique is used for cell counting, cell sorting, biomarker detection and protein engineering. Any suspended particle or cell from 0.2–150μm in size is suitable for analysis. Cells from solid tissue must be disaggregated before analysis.

It makes possible to determinate the intracellular mechanisms that lead to the inhibition of cellular proliferation. The evaluation of cellular viability to determinate the type of cell death can be achieved by the double stand with annexin V-FITC (AnV-FITC) and propidium iodide (PI). The evaluation of the cellular cycle to see its alterations is possible by evaluating content in DNA by labeling cells with PI. (Teixo 2013)
Protocol

Three different conditions have been analyzed to study cellular effects of HBOT, PDT and PDT+HBOT 60min compared to none treated colon cancer cells. The PS concentration used with PDT is 58.5nM as justified in previous work. (Laranjo 2014) Culture flasks with a growth area of 25cm$^2$ have been used to apply the correspondent therapy with 3 million cells. The culture medium is changed before incubation with PS. After 24h, before therapy, the culture medium is changed and cells are washed with PBS before the new medium is added to remove the excess of PS.

3.4.1 Analysis of cellular viability

Apoptosis is a normal genetically programmed process that occurs during embryonic development and in the maintenance of tissue homeostasis, under pathological conditions and in aging, where a cell actively participates in its own destructive processes. In apoptotic cells, the phospholipid phosphatidylserine membrane is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing it to the external cellular environment.

AnV-FITC is a protein with high affinity for phosphatidylserine and binds to its exposed apoptotic cell surface. That translocation precedes the loss of membrane integrity, which accompanies the later stages of cell death resulting from either apoptotic or necrotic processes.

AnV-FITC is typically used in conjunction with a vital dye such as PI for identification of early and late apoptotic cells and necrotic cells. The membranes of cells undergoing late apoptosis or necrosis are permeable to PI. This process is summarized in Figure 8.
The application of this double labeling with AnV-FITC and PI allows the following classification of cell population depending on its response: (BD Biosciences; Teixo 2013)

- **Group I**: viable cells or no measurable cell death, negative result for both markers.
- **Group II**: cells in early apoptosis with intact membranes, positive result for AnV-FITC and negative result for PI.
- **Group III**: cells in late apoptosis or necrosis, positive result for both markers.
- **Group IV**: cells in necrosis, positive result for PI and negative result for AnV-FITC.

**Protocol**

Cells obtained after trypsinization are placed in cytometry tubes and centrifuged again at 2500rpm. The supernatant is decanted and the resultant pellet is suspended in 100μL of a ligation buffer (1x). It is incubated 15min in dark at ambient temperature after the addition of 2,5μL of AnV-FITC and 1μL of PI (KIT Immunotech, Beckman Coulter, Check Republic) homogenized in a vortex. Subsequent to the incubation it is added 400μL of ligation buffer (1x) and it is analyzed in the cytometer using the wave lengths of 525nm for AnV-FITC and 640nm for PI.

The results of this technique are presented in percentage of cells from each group.

**3.4.2 Analysis of cell cycle**

This analysis allows determining the cell cycle status or tissue localization of individual cells within proliferating populations by using DNA dyes, as propidium iodide (PI), to verify the existence of alterations produced during the therapy given to the cells with HBOT and PDT.

The cell cycle consists in a sequence of events where one cell duplicates its genetic material and divides into two daughter cells that are genetically identical.
Cell growth, replication and division in eukaryotic cells occur according to a highly controlled series of events called cell cycle. The cell cycle is shown in Figure 9 and it has four main phases and: (BD Biosciences; Teixo 2013)

- During the first stage, **G1**, cells synthesize RNA and proteins to induce growth when the requisite signals of their environment are received. Corresponds to the first stage of the interphase.
- When there are proper conditions to cells can divide, cells enter the **S** stage of cell cycle and start DNA synthesis and replicate their chromosomal DNA. This phase belongs to the interphase.
- In the **G2** phase, cells continue to grow and prepare for mitosis. Is the last stage of the interphase.
- Mitotic phase, **M**, the mother cell divides into two genetically identical daughter cells.

![Cell cycle phases. (BD Biosciences)](image)

**Protocol**

Cells obtained after trypsinization are placed in cytometry tubes and centrifuged again during 5min at 1000rpm. The supernatant is decanted and it is incubated 30min in dark at 4°C after the addition of 200μL of ethanol 70% with the tube in agitation on the vortex. After the incubation 2mL of PBS (1x) is added and centrifuged at 2500rpm during 5min. The supernatant is decanted again and it is added 200μL of PI (PI/RNase Solution), homogenized in the vortex and incubated
15 min in dark at ambient temperature. Finally it is homogenized again and analyzed in the cytometer using the wave lengths of 488 nm for PI.

The results of this technique are presented in percentage of cells from each phase of the cell cycle.
CHAPTER 4: RESULTS

The results presented in this chapter were obtained for the metabolic activity evaluation and the two analyses of flow cytometry, cellular viability and cell cycle. All the experiments were performed in Biophysics Unit, Faculty of Medicine, University of Coimbra in collaboration with Ricardo Teixo and Ana Margarida Abrantes under supervision of Professor Maria Filomena Botelho.

4.1 Metabolic activity evaluation (MTT)

In order to evaluate the improvement of the photodynamic potential of PDT and HBOT therapies in different conditions, the assay of cytotoxicity was performed. With it, the level of metabolic activity can be analyzed in the colorectal cancer cell line (WiDr) after performing the different treatments approaches studied.

In order to obtain the EC$_{50}$ and R$^2$, the average and the standard deviation (SD) of the percentages of metabolic activity obtained by MTT assay, after the combined therapy, are calculated for 24h and 48h for each condition. Those values are shown in Table 5 and Table 6, which can be found in the Chapter 2: Detailed results of the Annexes, and they allow obtaining the curve of dose-response which is displayed in Figure 10 fitted with a sigmoid model. The final results for the values of EC$_{50}$ and R$^2$ are shown in Table 4.
Figure 10. Curve dose-response obtained by MTT assay displayed 24 and 48 hours after the therapies for all conditions (a) PDT + HBO 30min (b) PDT + HBO 60min (c) HBOT 30min + PDT (d) HBOT 60min + PDT.

It can be verified that preliminary results shown values of EC\textsubscript{50} for 24h after the treatments are very similar between all the treatments. The values for the treatments of PDT+HBOT 30min, PDT+HBOT 60min, HBOT 30min+PDT and HBOT 60min+PDT were 17,96nM, 18,43nM, 18,26nM and 19,65nM respectively. Between the values at 48h there is a tendency for a lower EC\textsubscript{50} value for the treatment HBOT 60min+PDT. The EC\textsubscript{50} obtained for the treatments PDT+HBOT 30min, PDT+HBOT 60min, HBOT 30min+PDT and HBOT 60min+PDT were 17,10nM, 15,98nM, 18,39nM and 7,76nM. However, it could be observed R\textsuperscript{2} values lower than 0,90 showing that further studies are needed.
Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment

Table 4. EC\textsubscript{50} and \( R^2 \) calculated with the values of Table 1 and 2 in ‘Chapter 2: Detailed results of the Annexes’ with the program OriginPro 8, for each condition after 24h and 48h of the corresponding treatment. (own source)

<table>
<thead>
<tr>
<th>MTT</th>
<th>Treatment</th>
<th>EC\textsubscript{50} (nM)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>PDT+HBOT 30min</td>
<td>17,95689</td>
<td>0,87295</td>
</tr>
<tr>
<td></td>
<td>PDT+HBOT 60min</td>
<td>18,42253</td>
<td>0,88356</td>
</tr>
<tr>
<td></td>
<td>HBOT 30min+PDT</td>
<td>18,2516</td>
<td>0,87703</td>
</tr>
<tr>
<td></td>
<td>HBOT 60min+PDT</td>
<td>19,64873</td>
<td>0,89535</td>
</tr>
<tr>
<td>48h</td>
<td>PDT+HBOT 30min</td>
<td>17,0464</td>
<td>0,72418</td>
</tr>
<tr>
<td></td>
<td>PDT+HBOT 60min</td>
<td>15,97038</td>
<td>0,56977</td>
</tr>
<tr>
<td></td>
<td>HBOT 30min+PDT</td>
<td>18,38926</td>
<td>0,49718</td>
</tr>
<tr>
<td></td>
<td>HBOT 60min+PDT</td>
<td>7,75226</td>
<td>0,32573</td>
</tr>
</tbody>
</table>

4.2 Flow cytometry studies

After verifying the influence of the combined therapies on the level of metabolic activity, it is needed to evaluate the type of cell death and the effects induced on the cell DNA after therapies.

The results obtained in the flow cytometry studies are shown for the four different conditions studied. For both cell viability and cell death as well for cell cycle studies, results are expressed as the mean percentage of cells with standard deviation also represented. The final results are available in Tables 7 and 8, which can be found in the Chapter 2: Detailed results of the Annexes.
4.2.1 Cellular viability

![Bar graph showing cellular viability](image)

**Figure 11.** Results of cellular viability assay. Type of cell death 24h after the treatment for each condition in WiDr cell line. Graphics obtained by the software GraphPad Prism.

The cellular viability and type of cell death percentages are presented in **Figure 11** obtained by double labeling with AnV-FITC and PI.

The number of viable cells maintains practically the same in control cells and HBOT, 70% and 61.67% respectively, but decreases with PDT and it is even more notable in the combination of PDT+HBOT 60min, 32.33% and 19% of viable cells. The percentage of cells in initial apoptosis does not present alterations with treatments when compared to control cells, being all the values between 9% and 15.33%. The same tendency of results is observed for cell death by late apoptosis/necrosis, being observed a slight increase from 4.67% to 12% of the HBOT alone treatment and PDT+HBOT 60min respectively. However, our preliminary results appear to present a tendency for an increase in cell death by necrosis. The percentage of cells in necrosis in control conditions is of 11.67%, and the application of therapies presents a tendency to an increase to 18.33% for HBOT alone, 45% for PDT and 60% for the combined therapy, in the combination of PDT followed by 60min of HBOT.
4.2.2 Cell cycle

In Figure 12 are displayed the results, in percentage, of the analysis with PI for each phase of the cell cycle.

The control cell culture without treatment does not have any percentage for the phase Pre-G0, when the others therapies show a percentage value between 1.89% and 6%. The therapies have not induced significant differences in the number of cells in the stages of G0/G1, S and G2/M. The percentages of HBOT has a more similar structure with the control, the percentage of cells in the stage S is bigger than cells in G2/M stage. On the other hand, PDT and PDT+HBOT 60min have bigger percentages of cells in the stage G2/M than S.

**Figure 12.** Results of cell cycle analysis. Phases of the cell cycle 24h after the treatment for each condition in WiDr cell line. Graphics obtained by the software GraphPad Prism. (own source)
CHAPTER 5: DISCUSSION

In order to evaluate the metabolic activity of the colon cancer cells after the combined therapies, MTT assay was performed to obtain EC$_{50}$ values, value commonly used to quantify the therapeutic effect of a therapy with drug (Laranjo 2014), and R$^2$, to have a reliability indication.

The values of EC$_{50}$ 24h and 48h after the treatments have a tendency to not shown any significant differences between. Treatment based on HBOT for 60min followed by PDT presents lower value at 48h after the treatment. Nevertheless, R$^2$ values do not reach 0,90 and just for the metabolic activity assay after 24h the values get close to 0,90, indicating that further studies are needed. Those values are shown in Table 4.

The treatment with a smaller EC$_{50}$ has clinical and economical advantages, as discussed earlier in this project, which are reduced adverse effects and low cost of the PS by reducing the quantity of PS used. Following this logic, the best treatment to consider should be HBO 60min+PDT, with an EC$_{50}$ of 7,76nM. However and as previously described, R$^2$ should be considered before this analysis.

Comparing those results with the ones obtained in Laranjo 2014 where PDT alone has been studied with the PS BBr2HPC in the human colorectal adenocarcinoma cell line WiDr, all the combinations of therapies had shown a lower EC$_{50}$ value than just the application of the photodynamic treatment with BBr2HPC
photosensitizing agent, as in the present study. The EC$_{50}$ for carcinoma colorectal after PDT treatment was 58,5nM for 24h after the treatment. (Laranjo 2014)

As the values of EC$_{50}$ maintain practically the same during the time after incubation considered (24h and 48h), we can assume that the combination of PDT and HBOT would cause irreversible damage in cells and thus, that there is no capacity of recovery after the alterations produced by the different treatments.

By using MTT assay we also verify that combination of PDT and HBOT induce a response dose-dependent, as increasing concentrations of PS induced increasing inhibition of metabolic activity in WiDr cell line, as is shown in **Figure 10**.

The metabolic activity assay can give some indication about the proliferative capacity of cells after the treatment but it does not show information about the intracellular mechanisms that leads to its cytotoxicity. For that reason it was used flow cytometry techniques, namely the double labeling with AnV-FITC and PI to assess the type of cell death induced by the treatments evaluated in this project, as well as the DNA marker PI to identify alterations in the cellular cycle.

Flow cytometry studies were performed 24h after each therapy alone in order to determine its cell effects compared to normal WiDr cells and for the treatment PDT+HBOT 60min. As time has not permitted start this second phase after having the complete results of metabolic activity, PDT+HBOT 60min was the best combined therapy at that point with an EC$_{50}$=10,12 and R$^2$=0,99.

It is important to determine if the cytotoxicity is produced by the mechanism of necrosis or apoptosis. Necrosis is detected for the loss of integrity of the cytoplasmatic membrane and its capacity of excluding PI and apoptosis for the high affinity of the protein AnV-FITC with phosphatidylserine of the membrane. The number of viable cells is considerably reduced in treatments where PDT is involved; however, the treatment with HBOT has a percentage of viable cells similar to the control, it just varies an 8,33%. That can be evidence about the non effect of HBOT as a stand-alone therapy. It has been observed that the percentage of cells in initial apoptosis and late apoptosis/necrosis remains without differences compared to control cells. The number of cells in late apoptosis/necrosis is slightly higher in the therapies with photodynamic therapy involved, PDT and PDT+HBOT 60min, but it does not imply any notable result. Nevertheless, it has been detected a tendency for an increment of cell death by
necrosis in PDT compared with the control and that difference is even bigger in the condition of PDT+HBOT 60min representing a 45%, 11.67% and 60% respectively.

Apoptosis is frequently called programmed cell death and proximately 10 million cells per day undergo apoptosis in a healthy adult human due to alterations. Such mechanism is needed to preserve the homeostasis of the organism and to control tissue size among others. The process of necrosis refers to a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents; considered an accidental and uncontrolled, non-programmed form of cell death whereby dramatic changes in crucial cell parameters of metabolism and cell structure take place, generally caused by chemical or physical injury. The consequences of necrotic and apoptotic cell death are quite different. In the case of necrosis, the inflammatory response may be caused by cytosolic constituents pouring into the intercellular space through the damaged plasma membrane; in apoptosis these products are safely isolated inside macrophages. Accumulating evidence shows that necrotic cell death is sometimes also controlled and programmed when a cell is unable to die by apoptosis. (Chaabane et al. 2013) An apoptotic response should be preferable in front of the necrotic response of colorectal cancer cells to the PDT and PDT+HBOT 60min treatment.

During the Pre-G0 phase the cell is not dividing. In G0/G1 cells produce RNA and synthesize protein and in the next phase, S, the DNA is duplicated. In the last stage, G2/M, cells grow and divide into two similar cell daughters. The obtained results do not show variance between the percentages of cells in each phase of the cell cycle. In spite of that, little differences can be noticed. The control does not have cells in the phase Pre-G0 and the other therapies do. The control and HBOT cells have a similar cell behavior, as PDT and PDT+HBOT 60min do. The first group has lower values in the stages G0/G1 and S and higher values in the phases Pre-G0 and G2/M than the second group, the therapies with photodynamic irradiation. It can indicate that treatments implying irradiation does not allow the cell duplication.

In this project, it has been determinate the therapy PDT+HBOT 30minutes the best treatment for colorectal adenocarcinoma with an EC$_{50}$=17.96nM and R$^2$=0.87 with an improvement compared to the treatment just with PDT, which had an EC$_{50}$=58.50nM. (Laranjo 2014) This treatment showed no capacity of
recovery after the alterations produced. The most percentage of cell death belongs to the process of necrosis and this therapy seems to not allow the cell duplication.
CHAPTER 6: CONCLUSION

Photodynamic therapy (PDT) has been accepted as an alternative treatment for cancer, especially for solid tumors like carcinomas. It has some advantages over the current cancer chemotherapy and radiation therapy in terms of less adverse effects and targeted delivery. PDT requires three elements: light, a photosensitizer and molecular oxygen; (Shao et al. 2012) and is able to induce cell death by oxidative stress through activation with light of a non-toxic photosensitizer. (Teixo 2013; Triesscheijn et al. 2006) The efficacy of PDT can be diminished because of regions of hypoxia, areas with a poorer oxygenation also present in colorectal cancer, suggested as an adverse prognostic factor for patient outcome. (Sudhakar et al. 2013; Vaupel and Mayer 2007) To alter the hypoxic state and improve treatment outcome it is used the hyperbaric oxygen therapy (HBOT). (Daruwalla and Christophi 2006; Ogawa et al. 2013)

Four possible treatments have been studied to improve PDT outcome in WiDr cell line: PDT+HBO 30min, PDT+HBO 60min, HBO 30min+PDT and HBO 60min+PDT. For PDT it has been used the photosensitizing agent BBr2HPC and irradiation with 10J, and for HBOT it has been applied, 1bar of 100% O₂. To study their outcome, metabolic activity assay with MTT have been performed 24h and 48h after the treatment revealing an improvement from EC₅₀=58,50nM of PDT treatment alone (Laranjo 2014) to EC₅₀=17,96nM of PDT+HBOT 60min, both assays 24h after the therapy. The value of R² does not reach 0,90 and more assays should be realized to have a better value for the fitting results and a
definitive decision about the best therapy, as the results for all the therapies analyzed 24h after the treatment are similar.

To analyze the cytotoxicity effects of the treatment, two flow cytometry analysis have been performed: cellular viability assay and cell cycle analysis. To compare the effects of the different therapies, 24h after the treatment four conditions have been subjected to study: control, PDT, HBOT and PDT+HBOT 60min. As mentioned before, the combined therapy analyzed should had been the best of the eight studied before, PDT+HBOT 30min 24h after the treatment. To confirm the cellular effects of the colorectal cancer therapy, both assays of flow cytometry should be repeated studying the therapy with better EC₅₀ and R². The cellular viability assay showed an increased percentage of necrotic cells, although that should be preferable apoptosis. In regard to cell cycle analysis, it has not shown differences but it can indicate not allow the cell duplication or in other words, the therapy does not allow colorectal cancer cell proliferate.

In order to assure the therapy conditions and effects, other assays can be performed in order to complement the analysis realized. Those assays could be Alamar blue to complement the metabolic activity results and assays to evaluate the therapy cytotoxicity such as comet assay and p53 protein expression.

When this first phase, in vitro studies, is completed a second phase of in vivo studies should be develop by making an orthotopic animal model of colorectal adenocarcinoma and evaluate the different therapeutics approaches in the orthotopic model. Finally, ex vivo studies by excising the tumors of the animal model and perform histological analysis and immunohistochemical studies and analyze the expression of VEGF and HIF-1 by western blot.

This project has highlighting the combined therapy hyperbaric photodynamic therapy as a promising therapeutic approach for adenocarcinoma colorectal cancer.
CHAPTER 7: BIBLIOGRAPHY

The scientific articles and websites consulted for the realization and completely understanding of this project are shown in the next pages.

7.1 Bibliographic references


Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment


7.2 Consulted bibliography


Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment


Annexes

“Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment”

DFP submitted to obtain the title of DEGREE in BIOMEDICAL ENGINEERING by Olga Ciutad Castejón

Barcelona, June 30th 2015

Director: Joan Francesc Alonso López
Escola Universitària d’Enginyeria Tècnica Industrial de Barcelona
Department (EUETIB)
Universitat Politècnica de Catalunya (UPC)
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CHAPTER 1: SOFTWARE

In this chapter are shown the different informatics programs used to obtain and evaluate the results with MTT.

1.1 Gen5 1.09

This software is used to obtain the values of absorbance in the wave length of 570nm and 620nm. The interface and the followed steps to acquire the results are shown bellow. Before starting with the absorbance readings, it must be assured that the spectrometer is connected and turned on. The plate has to be introduced without the cover. The process followed is shown in Figures 13-20.
Figure 13. Step 1 with Gen5. ‘Experiment’ is selected to open a new file. (own source)

Figure 14. Step 2 with Gen5. The kind of protocol of the new file is defined for MTT selecting ‘MTT Hugo.prt’. (own source)
Figure 15. Step 3 with Gen5. ‘Read plate’ is available with the button rounded in red. It will open the spectrometer tray allowing the reading of the plate. (own source)

Figure 16. Step 4 with Gen5. This window is shown when ‘Read plate’ is clicked and by selecting ‘Read’ it will permit the saving of the experiment. (own source)
**Figure 17.** Step 5 with Gen5. To continue, the experiment must be saved. (own source)

**Figure 18.** Step 6 with Gen5. The temperature is displayed on the screen to validate the experiment. Before the next steps, the plate must be in the tray. (own source)
Figure 19. Step 7 with Gen5. The spectrometer reads first the 570nm and when it is finished, the results are presented on the screen. By selecting the Excel icon, rounded in red, the results are exported to an Excel sheet. To visualize the 620nm results, 'Data' must be changed to '620', rounded in blue. (own source)

Figure 20. Step 8 with Gen5. When 620nm results are ready they appear on the screen and are exported in the same way as the 570nm results. They appear in the same Excel sheet. (own source)
1.2 OriginPro 8

This program allows determining the EC$_{50}$ and the R$^2$ by the percentages obtained by metabolic activity evaluation. The calculation of the concentration logarithm with base 10, the average of the percentages and the standard deviation (SD) of the group of results of MTT for each condition is needed to obtain the values of EC$_{50}$ and R$^2$. Those previous values are obtained with Excel as it is explained in the 3.3 MTT assay section of the Technical Report. The process followed is shown in Figures 21-25.

**Figure 21.** Step 1 with OriginPro 8. The X axe corresponds with the concentration logarithm with base 10, the average of the percentages corresponds with Y axe and the SD is set as Y error. (own source)
Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment

**Figure 22.** Step 2 with OriginPro 8. The values on the table are selected and a fit sigmoidal analysis is chosen as the image shows. (own source)

**Figure 23.** Step 3 with OriginPro 8. In ‘Function Selection’ a dose-response ‘DoseResp’ is selected as ‘Function’ and in the ‘Parameters’ section the top and bottom values of Y axe are set to 100 and 0 respectively. (own source)
Figure 24. Step 4 with OriginPro 8. A set of numeric values are displayed and among them the value of 'EC₅₀' and 'Adj. R-Square' ($R^2$) as marked in the image. (own source)

Figure 25. Step 5 with OriginPro 8. The dose response curve is obtained too with the tables shown on Figure 24. (own source)
CHAPTER 2: DETAILED RESULTS

The percentages obtained by the spectrometer for each concentration and condition are shown in Table 5 and Table 6 for 24h and 48h respectively. In Table 7 and Table 8 it is available the average and the SD for cellular viability and cell cycle assay.

Table 5. PS concentration logarithm with base 10, average and standard deviation for MTT assay displayed 24 hours after the therapies.

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Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment

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Table 6. *PS concentration logarithm with base 10, average and standard deviation for MTT assay displayed 48 hours after the therapies.*
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<td>HBO 60min+PDT</td>
<td>100,00</td>
<td>-</td>
<td>78,74</td>
<td>4,504809</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5nM</td>
<td>0,69897</td>
<td>78,74</td>
<td>4,504809</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15nM</td>
<td>1,176091</td>
<td>75,24</td>
<td>3,972903</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25nM</td>
<td>1,39794</td>
<td>3,10</td>
<td>1,007279</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50nM</td>
<td>1,69897</td>
<td>1,51</td>
<td>0,407247</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100nM</td>
<td>2</td>
<td>0,84</td>
<td>0,113968</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250nM</td>
<td>2,39794</td>
<td>0,80</td>
<td>0,058223</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500nM</td>
<td>2,69897</td>
<td>1,21</td>
<td>0,438694</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 7. Results for cell cycle assay 24h after the treatment. Average and standard deviation for each type of cell death, viable, initial apoptosis, late apoptosis/necrosis and necrosis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable</th>
<th>Initial Apoptosis</th>
<th>Late Apoptosis/Necrosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>SD</td>
<td>Average</td>
<td>SD</td>
</tr>
<tr>
<td>Control</td>
<td>70</td>
<td>5,20</td>
<td>11,67</td>
<td>2,52</td>
</tr>
<tr>
<td>PDT</td>
<td>32,33</td>
<td>10,69</td>
<td>11,67</td>
<td>1,53</td>
</tr>
<tr>
<td>HBO 60min</td>
<td>61,67</td>
<td>8,39</td>
<td>15,33</td>
<td>5,86</td>
</tr>
<tr>
<td>PDT+HBO 60min</td>
<td>19</td>
<td>5,29</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 8. Results for cell cycle assay 24h after the treatment. Average and standard deviation for each cell cycle phase, Pre-G0, G0/G1, S and G2/M.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-G0</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>SD</td>
<td>Average</td>
<td>SD</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>57,80</td>
<td>1,64</td>
</tr>
<tr>
<td>PDT</td>
<td>5,40</td>
<td>3,21</td>
<td>55,20</td>
<td>9,20</td>
</tr>
<tr>
<td>HBO 60min</td>
<td>1,86</td>
<td>0,69</td>
<td>59</td>
<td>6,27</td>
</tr>
<tr>
<td>PDT+HBO 60min</td>
<td>6</td>
<td>2,45</td>
<td>53</td>
<td>7,44</td>
</tr>
</tbody>
</table>
Budget

“Photodynamic therapy improvement with hyperbaric oxygen therapy for human colon cancer treatment”

DFP submitted to obtain the title of DEGREE in BIOMEDICAL ENGINEERING by Olga Ciutad Castejón

Barcelona, June 30th 2015

Director: Joan Francesc Alonso López
Escola Universitària d’Enginyeria Tècnica Industrial de Barcelona
Department (EUETIB)
Universitat Politècnica de Catalunya (UPC)
BUDGET - INDEX

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CHAPTER 1: BUDGET

This budget details the costs of the elaboration of this project. Note that the most expensive concept in the budget is personnel costs, due to the reusable laboratory equipment during several years.

1.1 Materials budget

The materials budget is divided in laboratory equipment, computer and software and office costs shown in Tables 9-12. Those costs are calculated for the period of time that one academic semester last, corresponding to a total of 16 weeks of work.

Other laboratory equipment used in the current research but acquired years ago, like Binder incubator, vertical laminar flow cabinet (Holten Laminair HB2448), PDT fluorescent and red filter, centrifuge 40mL, vortex, flux cytometer, spectrometer (Biotek Synergy HT multi-mode microplate reader) and Vacusafe comfort aspiration system, and other laboratory material, as pipette P20, pipette P10, repetitive pipette, pipette 3mL and test tubes rack, allows a saving in the project budget of almost 26.000€.
### Table 9. Laboratory costs.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Fixed cost (€/unit)</th>
<th>Number of units</th>
<th>Total (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Laboratory equipment</strong></td>
<td></td>
<td></td>
<td><strong>2,047,75 €</strong></td>
</tr>
<tr>
<td>Pressure cooker (Silampos Lagos)</td>
<td>69,90</td>
<td>1</td>
<td>69,90</td>
</tr>
<tr>
<td>O₂ tank 680L</td>
<td>1.962,90</td>
<td>1</td>
<td>1.962,90</td>
</tr>
<tr>
<td>Security valve</td>
<td>14,95</td>
<td>1</td>
<td>14,95</td>
</tr>
<tr>
<td><strong>Biological and chemical material</strong></td>
<td></td>
<td></td>
<td><strong>1,687,35 €</strong></td>
</tr>
<tr>
<td>WiDr cell line</td>
<td>575</td>
<td>1</td>
<td>575</td>
</tr>
<tr>
<td>Dulbecco's Modified Eagle's Medium (DMEM, Sigma D-5648) 10L</td>
<td>30,20</td>
<td>1</td>
<td>30,20</td>
</tr>
<tr>
<td>Fetal bovine serum (Sigma F7524) 50mL</td>
<td>20,38</td>
<td>1</td>
<td>20,38</td>
</tr>
<tr>
<td>Sodium pyruvate (Gibco 11360) 100mL</td>
<td>11,50</td>
<td>1</td>
<td>11,50</td>
</tr>
<tr>
<td>Antibiotic Antimycotic Solution (100×), Stabilized (Sigma A5955) 20mL</td>
<td>15,20</td>
<td>1</td>
<td>15,20</td>
</tr>
<tr>
<td>Phosphate buffer saline 200mL (PBS)</td>
<td>77,50</td>
<td>1</td>
<td>77,50</td>
</tr>
<tr>
<td>Polyethylene glycol 400 (PEG400) 1L</td>
<td>17,12</td>
<td>1</td>
<td>17,12</td>
</tr>
<tr>
<td>Ethanol (EtOH) 475mL</td>
<td>11,65</td>
<td>1</td>
<td>11,65</td>
</tr>
<tr>
<td>Thiazolyl blue or MTT 500mg</td>
<td>45,60</td>
<td>1</td>
<td>45,60</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl) 100mL</td>
<td>36,70</td>
<td>1</td>
<td>36,70</td>
</tr>
<tr>
<td>Isopropyl alcohol (2-propanol) 100L</td>
<td>34,40</td>
<td>1</td>
<td>34,40</td>
</tr>
<tr>
<td>Item</td>
<td>Quantity</td>
<td>Unit Cost</td>
<td>Total Cost</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
<td>-----------</td>
<td>------------</td>
</tr>
<tr>
<td>Buffer solution (1x) 1L</td>
<td>1</td>
<td>19.77</td>
<td>19.77</td>
</tr>
<tr>
<td>Annexin V-FITC Apoptosis Detection Kit (100 tests)</td>
<td>1</td>
<td>490</td>
<td>490</td>
</tr>
<tr>
<td>Ethanol 70% 500mL</td>
<td>1</td>
<td>7.33</td>
<td>7.33</td>
</tr>
<tr>
<td>Cell Cycle Assay Kit (100 tests)</td>
<td>1</td>
<td>295</td>
<td>295</td>
</tr>
<tr>
<td><strong>Laboratory material</strong></td>
<td></td>
<td>984.73 €</td>
<td></td>
</tr>
<tr>
<td>Latex gloves (50 pairs)</td>
<td>4</td>
<td>18.20</td>
<td>72.8</td>
</tr>
<tr>
<td>Sterile 24 well plate (100 units)</td>
<td>1</td>
<td>103</td>
<td>103</td>
</tr>
<tr>
<td>Sterile 96 well plate (100 units)</td>
<td>1</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Sterile culture flasks 25cm² (200 units)</td>
<td>1</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td>Test tubes (200 units)</td>
<td>1</td>
<td>57.40</td>
<td>57.40</td>
</tr>
<tr>
<td>Pipette tips 0,5-20 μL (96 units)</td>
<td>1</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Pipette tips 0,1-10 μL (96 units)</td>
<td>1</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Precision Dispenser Tips 10mL (100 units)</td>
<td>1</td>
<td>128.90</td>
<td>128.90</td>
</tr>
<tr>
<td>Eppendorf tube 5mL (50 units)</td>
<td>1</td>
<td>43.20</td>
<td>43.20</td>
</tr>
<tr>
<td>Centrifuge tubes 50mL (100 units)</td>
<td>1</td>
<td>72.50</td>
<td>72.50</td>
</tr>
<tr>
<td>Parafilm (4in. × 125ft)</td>
<td>1</td>
<td>37.10</td>
<td>37.10</td>
</tr>
<tr>
<td>Wash bottle screw caps</td>
<td>1</td>
<td>1.49</td>
<td>1.49</td>
</tr>
<tr>
<td>Cellulose bobbin (2 rolls)</td>
<td>1</td>
<td>16.34</td>
<td>16.34</td>
</tr>
<tr>
<td><strong>Gross total</strong></td>
<td></td>
<td>4719.83 €</td>
<td></td>
</tr>
<tr>
<td><strong>IVA (21%)</strong></td>
<td></td>
<td>991.17 €</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>5711 €</td>
<td></td>
</tr>
</tbody>
</table>
Table 10. Computer and software costs.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Fixed cost (€/unit)</th>
<th>Number of units</th>
<th>Total (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gen5 1.09 (undefined)</td>
<td>2.510,57</td>
<td>1</td>
<td>2.510,57</td>
</tr>
<tr>
<td>OriginPro 8 (1 year license)</td>
<td>1.168,71</td>
<td>1</td>
<td>1.168,71</td>
</tr>
<tr>
<td>Acer Laptop ES1-512-P6EL</td>
<td>329,99</td>
<td>1</td>
<td>329,99</td>
</tr>
<tr>
<td>Microsoft Office 365 (1 year license)</td>
<td>69,99</td>
<td>1</td>
<td>69,99</td>
</tr>
<tr>
<td><strong>Gross total</strong></td>
<td></td>
<td></td>
<td><strong>4.079,26</strong></td>
</tr>
<tr>
<td>IVA (21%)</td>
<td></td>
<td></td>
<td><strong>856,64</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>4.935,90</strong></td>
</tr>
</tbody>
</table>

Table 11. Office costs.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Cost (€/month)</th>
<th>Months</th>
<th>Total (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electricity bill</td>
<td>100</td>
<td>5</td>
<td>500</td>
</tr>
<tr>
<td><strong>Gross total</strong></td>
<td></td>
<td></td>
<td><strong>500</strong></td>
</tr>
<tr>
<td>IVA (21%)</td>
<td></td>
<td></td>
<td>105</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>605</strong></td>
</tr>
</tbody>
</table>
1.2 Personnel budget

The costs are calculated for the period of time that the investigation project lasted, 5 months. The total cost is shown in Table 12.

Table 12. Personnel costs.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Cost (€/h)</th>
<th>Hours</th>
<th>Total (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Student</td>
<td>10</td>
<td>800</td>
<td>8.000</td>
</tr>
<tr>
<td>Supervisor</td>
<td>18</td>
<td>900</td>
<td>16.200</td>
</tr>
<tr>
<td>Project director</td>
<td>30</td>
<td>1.000</td>
<td>30.000</td>
</tr>
<tr>
<td><strong>Gross total</strong></td>
<td></td>
<td></td>
<td>54.200</td>
</tr>
<tr>
<td><strong>IVA (21%)</strong></td>
<td></td>
<td></td>
<td>11.382</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>65.582</td>
</tr>
</tbody>
</table>
1.3 Global budget

*Table 13. Total cost of the project, IVA included.*

<table>
<thead>
<tr>
<th>Concept</th>
<th>Total (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory costs</td>
<td>5.711</td>
</tr>
<tr>
<td>Computer and software costs</td>
<td>4.935,90</td>
</tr>
<tr>
<td>Office costs</td>
<td>605</td>
</tr>
<tr>
<td>Personnel costs</td>
<td>65.582</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>76.833,90</strong></td>
</tr>
</tbody>
</table>

The total cost of the project is 76.833,90€ as it is indicated in *Table 13.*