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Isolation and characterization of PDT-resistant cancer cells

Alicia Zamarrón[ad], Silvia R. Lucena[ad], Nerea Salazar[ad], Francisco Sanz-Rodríguez[ad], Pedro Jaén[bd], Yolanda Gilaberte[cd], Salvador González[bd], Ángeles Juarranz[ad]

[a]Department of Biology, Facultad de Ciencias, Universidad Autónoma de Madrid. Cantoblanco 28049, Madrid, Spain.

[b]Department of Dermatology, Hospital Ramón y Cajal, Madrid, Spain.

[c]Department of Dermatology, Hospital San Jorge, Huesca, Spain.

[d]Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain.

Address for correspondence:

Dr. Ángeles Juarranz, Darwin 2, Universidad Autónoma de Madrid, Cantoblanco (28049), Madrid, España. Tel.: +34-91-4978247. Fax: +34-91-4978344.

e-mail: angeles.juarranz@uam.es

Abstract

Even though the efficacy of Photodynamic therapy (PDT) for treating premalignant and malignant lesions has been demonstrated, resistant tumor cells to this therapy occasionally appear. Here, we describe the published methods to isolate resistant cancer cells to PDT and propose new procedures that may be used, as laboratory models allow a better understanding of resistance mechanisms. For this purpose, the treatment conditions, the photosensitizer (PS) or pro-drug, the cell line and the final selection- clonal of total population- must be taken into account. In general, high and repeated treatment doses are used. The resistant cell population characterization may include cell morphology, response to PDT, expression of death proteins or survival related genes and cell proliferation analysis. In addition, *in vivo* models such as resistant cell transplantation to mice permit evaluating tumorigenicity and aggressiveness, leading to the determination of the *in vivo* resistance. Summarizing, in order to improve clinical results, cellular models can help to understand PDT-resistance mechanisms *in vivo* and *in vitro*.

Key Words: Photodynamic therapy; Photosensitizer; Resistance; Isolation; Tumor cells

Abbreviations: ALA, δ-aminolevulinic acid; ALDH1, aldehyde dehydrogenase 1; ABC, ATP-binding cassette; BCC, basal cell carcinoma; BCRP, breast cancer resistant protein; BPD-MA, benzoporphyrin derivative monoacid ring A; CAM, cell adhesion molecule; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinases; HPPH, 2-(1-hexyloxethyl)-2-devinyl pyropheophorbide-a; IAP, inhibitor of apoptosis protein; MAL, methyl δ-aminolevulinic acid; MAPK, mitogen-activated protein kinase; MDR, multidrug resistance; MRP, multidrug resistant associated protein; NMSC, non melanoma skin cancer; PHP, polyhematoporphyrin; P-gp, P-glycoprotein; PDT, photodynamic therapy; PpIX, protoporphyrin IX; PS, photosensitizer; PII, photofrin II; PPC, Zn(II) pyridinium-substituted phthalocyanine; SOD, superoxide dismutase.

Background

Anticancer therapies effectiveness is principally limited by resistance. Failure in chemotherapy and radiotherapy treatments leads to tumor progression and poor clinical prognosis [1-4]. Although, the resistance process has been extensively studied in both therapy modalities it is still poorly understood. Photodynamic therapy of cancer (PDT), may induce resistance in patients too - even though it is not well documented [5-8].

In vitro and *in vivo* models, based on selecting resistance cells, allow the researches to gain a better understanding of the resistance process. Models of head, neck, lung, esophagus, urinary bladder, gynecological cancers and, non-melanoma skin cancer (NMSC) are being used for resistance to PDT research [9-12]. In order to analyze the mechanisms underlying PDT-resistance, these cancer models are very useful to perform systematic, molecular and functional studies.

Several factors, besides tumor origin, must be taken into account when developing an appropriate resistant cancer model- the way of acquiring the resistance and the characteristics of the tumor. As a rule, the first cycle of treatment kills most tumor cells. However, some of them could not react properly to the therapy and become more aggressive after several cycles of treatments.

On the whole, resistance can be classified in two different categories: (i) intrinsic, if resistance-mediating factors already existed in the tumor cells before the treatment, and (ii) acquired, if it arises as a consequence of the treatment in responsive tumors. Intrinsic resistance is due to a complex set of molecular and biochemical properties of the tumor, which lead to cell death avoidance. Nevertheless, acquired resistance can be produced by different factors such as a

limited amount of drug, hypoxia or scarce radiation reaching the tumor, as well as those that affect the micro-environment and the mutations that appear during the treatment [13-16]. Other adaptive responses, for instance, the activation of alternative compensatory signaling pathways, have also to be considered.

Moreover, tumors can present a high molecular heterogeneity with a large number of genotypic and phenotypic variations [17-19]. Therefore, different areas of a tumor might have dissimilar properties as well as diverse sensitivity degrees to therapies. This can particularly correlate to acquired resistance, by which tumors not only become resistant to a particular therapy, but they may also develop cross-resistance to other therapies. For instance, resistance to multiple drugs is quite evident in chemotherapy.

Hence, resistance can appear due to the selection of a cell subpopulation that already had resistant characteristics in the original tumor, or/ and a cell population that has developed resistance to that therapy after the treatment. Regarding to PDT, in both situations, the therapy can leave a significant percentage of surviving cells that have not been destroyed due to the insufficient quantity of reactive oxygen species (ROS) produced when the light excites the photosensitizer (PS). Therefore, changes that may have occurred in migration, division, cell death or the appearance of mutations and could have direct effects on the response to a new photodynamic treatment and on the global tumor growth.

In this context, we describe below diverse published methods to isolate PDT-resistant tumor cells as well as some of its particular characteristics. Such methods facilitate the study of long-term cellular, biochemical and molecular changes induced by the treatment. A better understanding of

PDT action mechanisms will permit to adapt the general PDT protocols or to choose other therapies in order to enhance the clinical management of a specific type of cancer.

Cellular Resistance Models

Chemotherapy researchers have generated drug-resistant cell models in the laboratory mainly by exposing repetitively a cancer cell culture to increasing concentrations of drug. Afterwards, parental cells have being compared to the resistant population employing diverse assays (e.g. viability/proliferation assays, such as the MTT, or the clonogenic assay) [20-22].

In 1960, chemotherapeutic agents- methotrexate, vinblastine and terephthalanilide- were used to develop in mouse models different drug-resistant cells lines [20]. Although, it was not until 1970 that the development of cancer cell lines resistant to chemotherapy was described [23]- researchers selected cell lines from chinese hamsters after repeated treatments with an increasing dose of actinomycin D. Some of such cells presented a 2500-fold higher drug-resistance than parental cells. In addition, resistant cell lines were cross-resistant to other chemotherapeutic drugs such as vinblastine and doxorubicin. Since then, many different drug-resistant cells have been isolated by repeated treatments with a chemotherapeutic agent.

This strategy has been employed so as to isolate resistant cells to other therapies. For example, in 1988, Hahn and van Kersen [24] isolated heat-resistant cell strains from fibrosarcoma mouse cells generated by radiation (RIF-1), after 11 cycles of heating (60 min at 45ºC) and regrowth of the surviving cells to the previous heating cycle. Furthermore, they selected diverse resistant strains derived from single surviving cells. This resistant strains do not exhibited changes on growth rate or plating efficiency in comparison with parental RIF-1 cells, as well as they did not present morphological abnormalities.

This approach of applying repeated cycles of treatment has been used to generate PDT-resistant cells as well. With this aim, resistant cells have been obtained by the administration of exogenous PSs, such as Photofrin or phthalocyanines, and endogenous ones, for instance, protoporphyrin IX (PpIX)- formed from two different precursors δ-aminolevulinic acid (ALA) or methyl δ-aminolevulinic acid (MAL), through the heme biosynthetic pathway [25-27].

Once the resistant cells are identified and isolated, in order to calculate the Fold Resistance Index, tumor cells are exposed to specific treatment conditions followed by cell viability assays. The main index used in chemotherapy to determine the resistance degree is the half maximal inhibitory concentration (IC50)- the concentration of a drug that is required for 50% inhibition *in vitro*.

IC50 = Resistant Cell Line IC50 / Parental Cell Line IC50

However, in PDT, it does not exist a good definition of a Fold Resistance Index. It is usually referred to determined selected treatment conditions- PS concentration and dose light irradiation- that induce lethality, for instance, of 50% (LD50) or 90% (LD90) in the parental cell line:

Fold Resistance = Resistant Cell Line (LD50 or LD90) / Parental Cell Line (LD50 or LD90)

In order to establish the possible level of resistance in clinic, it would be an ideal situation to work with cell cultures directly established from a tumor of a patient, comparing them before and after the treatment. McDermott et al. [20] have recently summarized data from chemotherapy-resistant cell lines isolated from patients with lung, neuroblastoma and ovarian cancers, and they indicate that the majority of the cell lines showed from 2 to 5-fold increase on their resistance to the drug in relation to the parent cell line, based on their IC50.

Nevertheless, in PDT, the resistance fold increase is not so high, so it is considered a variant as resistant when it has a fold increase equal to or greater than 1.5 over parental cells [25,26]. In this therapy, after selecting the treatment conditions that induce a LD50 or LD90, (drug concentration and light dose) tumor cells are subjected to repeat PDTs to isolate completely resistant populations or specific resistant clones from the original mixed population.

To sum up, the main objective is to develop a laboratory model based on applying repeated therapy to obtain cells highly resistant *vs* parental cells, with a stable resistant resistance phenotype. To create an appropriate model some factors must be taken into account, including the parental cell line and the conditions of the treatment- light dose and concentration of drug- that should be optimized for each specific case. The recovery rate after the treatment is relevant, as even in identical conditions there might be differences between PSs. The resistant line has to be able to return to the exponential growth phase thereby ensuring the selection of resistant cell subpopulations.

Strategies for the Selection of Resistant Cells

In order to reproduce the resistance after therapy in preclinical studies, validated protocols are based on *in vitro/in vivo* selection of cancer cells with intrinsic or acquired resistance after chronic treatment. There are multiple ways to select resistant cells to PDT and here we describe just a few of them.

In Vitro Selection

Cellular and molecular studies in cancer research about the bases of intrinsic and acquired resistance to therapies, PDT among others, could be performed by using mainly two *in vitro*

models: (i) primary cell cultures obtained directly from a tumor, whose resistance or sensitivity must be evaluated; and (ii) immortalized cancer cells lines with or without intrinsic resistance.

When choosing the first option, is preferable to select a cell culture not previously treated since the treatment may induce changes in the tumor cell behavior; for example, by increasing the expression of drug resistance markers, not related to the therapy being studied. However, cell lines derived from untreated human tumors are relatively rare.

In contrast, many immortalized cell lines are available for each cancer type, with different genetic alterations, what allows choosing the most accurate model to investigate *in vitro* the PDT-resistance mechanisms. In chemotherapy, two principal sources might be checked - the Genomics of Drug Sensitivity in Cancer (GDSC, www.cancerRxgene.org), which is the largest public resource for information on drug sensitivity in cancer cells and drug response molecular markers; and the Cancer Cell Line Encyclopedia (CCLE, www.broadinstitute.org/ccle) which includes data related with gene expression or chromosome copy number [28,29].

Nonetheless, scarce research in PDT-resistant cells selection has been made. In fact, PDT resistant cells have never been isolated from patients, while it is usually done to obtain resistant cells to chemotherapeutics. Therefore, selection of PDT-resistant cells has been carried out with immortalized cell lines including the cells coming from a radiation-induced fibrosarcoma in mice (RIF-1) [25,30]; murine mammary adenocarcinoma (LM3) [27]; human colon adenocarcinoma (HT29) [31]; human lung adenocarcinoma (CL1-5), human melanoma (A435) and human breast carcinoma (MDA-MB-231) [32]; and human squamous cell carcinoma (SCC-13) [7,33].

Apart from the cells (primary or established cell line) chosen for the isolation of the resistant cells in *in vitro* systems, it should be taken into account that tumors are heterogeneous.

Therefore, the cancer cell lines obtained from them would have also different features [17-20]. Nevertheless, there are three basic selection strategies for isolating anticancer therapy resistant cells (Figure 1), by selecting: (i) a resistant population from the original culture, (ii) resistant clones and (iii) cells with specific molecular markers.

1. Selecting a resistant population from the original culture

Firstly, a cell population is treated, and a small percentage of cells could resist, being them responsible of the repopulation of the culture. Then, this resistant population is subjected again to a new treatment and the surviving cells selected again. Probably, these surviving (resistant) cells would be heterogeneous and differ from the original parental cells, due to the appearance of genetic or epigenetic alterations that promote their survival [20,34,35]. In this sense, heterogeneity has been seen in actinomycin D or taxane-resistant models developed from human lung cancer cell lines [21,23,34,35] and in human breast cancer cells [22]. Besides the emergence of changes that enhance cell survival, there is also the possibility that the isolated cell population already had a resistant signature in the original culture. Indeed, this has been demonstrated for many drug-resistant models, which are often enriched in cancer stem cells (CSCs) markers [1,36-38]. CSCs are thought to be responsible for tumor regeneration after chemotherapy and radiotherapy and they could also have a role in resistance to PDT (39-41).

Therefore, it can be considered that from a heterogeneous group of initial cells, PDT would positively select those cells that possess intrinsic resistance mechanisms or promotes the development of determined genetic alterations. Cells without such mechanisms would die after the treatment. Moreover, the therapy could change the expression of molecules promoting the process of resistance. Given that the resistant cells can be heterogeneous, they could be also

selected, for instance, by cell sorting, using specific molecular cell markers, or by selecting specific clones.

2. Resistant clones

The resistant clones are selected by limited dilution in this method of isolation. Clonal selection has a major advantage; the isolated cells could be more resistant to the treatment than other clones from the same original cell line [20,42,43]. However, this method has also certain disadvantages, as that such clones are not necessarily the responsible for tumor relapse and, eventually, for metastasis.

There are two possible strategies within this method of isolation. The first one is based on the selection of resistant clones, after several rounds of treatments, just at the end of the process. Using this option it can also be investigated the heterogeneity within the developed drug-resistant model. In this sense, two cisplatin-resistant clones, obtained from a human colon cancer cell line (LoVo), were selected [43]. The clones showed distinct characteristics; one of the clones overexpressed the ABC transporter P-glycoprotein, whereas the other clone did not. Similar heterogeneity has also been described in cisplatin-resistant models developed from a human pancreatic cancer cell line with a mutation in the DNA repair protein BRCA2 [44].

In the second method, the resistant clones are selected after one cycle of treatment, and then they are subjected to a second cycle of therapy and again the most resistant clones are selected. These can be again subjected to new treatments. Clones can be collected from each round of the selection process. For instance, this protocol was used in the isolation of colchicine resistant cells, from the carcinoma cell line KB3-1 treated with three stepwise increasing drug treatments [42].

3. Cells with specific molecular markers

Resistant cells to cancer therapies can express determined differential molecular markers, and this feature can be used for their isolation. For an effective cell selection, a combination of different markers in the resistant cells might be used, because, both intrinsic and acquired resistance to anticancer therapies, result from numerous genetic and epigenetic changes. Since resistance to chemotherapy occurs at different levels, including activation of oncogenes, inhibition of tumoral suppressors, variations in drug influx/efflux or apoptosis evasion, different markers can be employed to identify them [14,45,46]. In addition, stem cell characteristics are also important factors in the resistance process [1,36]. In the case of PDT, all these factors could be implicated in promoting resistance. Alterations in the expression of many different genes have been observed and, therefore, multiple signaling pathways may contribute to PDT resistance [11,47,48].

The ATP-binding cassette (ABC) transporter family increases drug efflux and, thus, reduces the intracellular drug concentration; participating in drug resistance. Within this large family of proteins, P-glycoprotein (MDR, Pgp or ABCB1), multidrug resistance protein 1 (MRP1 or ABCC1) and ABCG2 are the most frequently associated with multidrug resistance. The expression levels of these proteins are variable in cancer cells [49]. Accordingly, ABCG2 is being used as an important marker for selecting MDRs cancer cells. ABCG2 can bind and efflux a wide range of structurally different classes of PS used both preclinically and clinically, such as porphyrins and chlorins [50]. It is expressed at different levels on cell lines that are used for many *in vitro* and *in vivo* PDT-models in which may affect the phototoxic efficacy [40]. Among the PSs that are substrates of ABCG2 there are included Photochlor, Benzoporphyrin derivative monoacid ring A (BPD-MA, Verteporfin), Hypericin and Protoporphyrin IX (PpIX). ABCG2

may reduce the PS intracellular levels below the threshold needed for cell death in tumors treated with PDT, thus leaving resistant cells that would probably repopulate the tumor [50,51].

Alterations in multiple signaling pathways can contribute to drug resistance. In this sense, another important factor is the epidermal growth factor receptor (EGFR). Modifications in this protein lead to a sustained activation of the MAPK/ERK signaling pathway. This change has been seen in several human malignancies including skin, colorectal, ovarian, breast, and prostate cancers, and often correlates with the enhanced cellular proliferation and development of cancer metastasis [52,53]. Therefore, it is an important potential factor in the resistance to PDT. In general, in cells with a good response to PDT, down-regulation of EGFR has been noted in *in vitro* and *in vivo*-treated cells. Besides it has been suggested that the decreased cell migration and the invasiveness in RIF-1-PDT-derived variants are related to the downregulation of EGFR. Compared to parental CL1-5, A375 and MDA-MB-231 cells, ALA-PDT caused a reduction in the level of EGFR in treated variants, which correlated with the reduced migration and invasion observed [32]. On the other hand, a recent study using A-431 squamous cell carcinoma of the skin and WiDr colorectal adenocarcinoma cells, linked EGFR and ERK activation as potential predictive factors of response to PDT [54]. The up-regulation of EGFR has been also demonstrated in patients with a bad response to PDT, as well as in the resistant PDT-SCC-13 cells [7].

Recently, CSCs have been identified in several cancers and have been proposed to explain the metastatic capacity, recurrence, and resistance to radiotherapy and chemotherapy. Several evidences suggest that tumors contain a small subpopulation of cells that exhibit self-renewal capacity, proliferate infrequently, express several pluripotency genes and are responsible for tumor maintenance and metastasis. Anticancer agents that kill rapidly growing tumor cells do not

affect CSCs, therefore these need to be killed upon treatment to eradicate the tumor. If some, even a few, are left intact, they will be responsible for tumor drug resistance and relapse [36-38, 55].

Some markers have been associated to CSC and these can be used to select just these particular cells. For instance, a CD44+ cell population possesses the properties of CSCs in head and neck squamous cell carcinoma; and ABCG2 and aldehyde dehydrogenase 1 (ALDH1) activity have also been reported to identify CSCs cancer stem cells in several cancer types. In breast cancer, the stem cell population is CD44+/CD24 while CD133 marks cancer stem cells in brain tumors, colorectal and pancreatic carcinomas [56-58].

CSCs can be identified and isolated using different approaches including flow cytometry and magnetic-associated cell sorting. Moreover, recently, Adhikary et al. [59] selected a cell population from the squamous cell carcinoma SCC-13 and A-431 cell lines by using as marker the aldehyde dehydrogenase 1 (ALDH1), indicating that these cells with stem cell-like properties enhance its potential of developing a tumor. This could be an excellent approximation to evaluate the resistance of CSC to cancer therapies, including TFD. In this sense, Bostad et al. [39] selected cells with CSC markers (CD133) and found that these cells were resistant to PDT.

Examples of in vitro Selection of resistant cells to PDT

The generation of resistant cells variants to photodynamic therapy will enable to understand the molecular mechanisms of sensitivity to several PSs, based on inherent and induced resistance in different cell lines. PDT-resistant cell lines have been obtained by using various photosensitizers such as Photofrin, phthalocyanines, Nile Blue or protoporphyrin IX, among others.

Luna and Gomer [25] performed the first studies of isolation of PDT-resistant cells. From the mouse radiation-induced fibrosarcoma (RIF-1) cell line, they isolated PDT-resistant variants following a protocol of repeated porphyrin (Photofrin II, PII) incubation and light treatments. They used two incubation procedures, and depending on the incubation time, 16 h (extended period) or 1 h (short period), the intracellular photosensitizer localization in the cells was different. They selected two individual colonies from each PDT porphyrin incubation time used by cloning. Clones had different behaviour and morphologic characteristics. More specifically, all resistant variants had increased protein content and were larger than the parental RIF-1 cells.

However, *in vitro* growth rates were similar. Flow cytometric analysis, by using propidium iodide, showed the characteristic mixture of diploid and tetraploid subpopulations for the parental and for one of the selected clones, whereas, a complete tetraploid phenotype was present in the other three PDT-resistant variants.

In the same way, using the RIF-1 tumor cells, Singh et al. [26] induced resistant populations to PDT by repeated treatments with PII (4 or 18 h of drug incubation) up to a 0.1-1 % survival level, followed by regrowth of single surviving colonies. When exposed to increasing PII concentrations, 18 h of drug incubation and fixed light exposure; the resistance was shown as the increased cell survival in the strain designated as RIF-8A, compared to the wild-type RIF-1 cells. The same authors also evaluated the resistance to PDT in Chinese hamster ovary-multidrug resistant (CHO-MDR) cells, compared to the CHO wild type cells. These findings suggest that different mechanisms are responsible for PDT-induced resistance and multi-drug resistance.

Lately, the same researches, by using three different photosensitizers (aluminum phthalocyanine tetrasulfonate, AlPcS4; Nile Blue A and Photofrin) with different localization properties, induced

distinct resistant populations in three human cell lines: neuroblastoma (SK-N-MC), human colon adenocarcinoma (HT29) and human bladder carcinoma (HT1376) [31]. Cells were incubated for 1 h (Nile Blue) or 18 h (AlPcS4 and Photofrin) using two drug concentrations and two different light doses. They evaluated the cell survival by the colony-forming assay and the authors indicated that multiple cultures were performed from single surviving colonies. Cells were regrowth and treated again receiving between 8 and 14 PDT cycles. Each treatment cycle was aimed to achive survival levels between 1-10%. They considered as PDT-resistant variants those cells with over 1.5-fold increase in PDT resistance. Resistant cells were then isolated by the colony forming assay. They obtained several resistant cell lines from HT29 using the three PSs and from HT1376 using the PS Nile Blue. The isolated clones obtained from HT1376 with AlPcS4 or Photofrin and those from SK-N-MC with any of the three PSs did not show resistance to PDT. All the cell lines showed different levels of intrinsic resistance. As the authors indicate, the variability in sensitivity to a single photosensitizer between cell lines is not surprising. However, the resistance relative rankings are very interesting and highlight the importance of an appropriate photosensitizer selection. The authors suggest that a specific variation within the population or a selectively advantageous mutation during the repeated treatments facilitates the development of resistant variants.

Casas et al. [27] isolated resistant clones of murine adenocarcinoma cells (LM3) after repeated ALA-PDT treatments. The authors used a concentration of 0.6 mM of the pro-photosensitizer ALA and varied the light doses (0.36 - 5.4 J/cm^2) to achieve survival levels between 5-10 %. The surviving cells were grown and were again subjected to a new cycle of ALA-PDT. The final population had received a total of 13 cycles (LM3L13) and, afterwards, 8 clones were isolated by the limiting dilution method. The LD50 was defined as the light dose that killed 50% of the cells

at saturating concentrations of ALA. The resistance index to ALA-PDT was defined as LD50 resistant clone/LD50 LM3. In both cases, the resistant clones isolated showed a stable level of resistance.

On the other hand, Mayhew et al. [30], using polyhematoporphyrin (PHP) and Zn(II) pyridinium-substituted phthalocyanine (PPC) as PSs, isolated two RIF-1 resistant cell populations, that demonstrated a 5.7 and 7.1-fold increase in resistance, respectively. Both resistant strains were isolated after 15 cycles of PDT with increasing sensitizer concentrations and fixed light doses. After the photosensitization cycles, the isolated strains were: RIF-25R from PHP treatment, and P10 strain from PPC treatment.

Milla et al. [33] developed PDT-resistant cells by using a cell line obtained from a squamous cell carcinoma of skin (SCC-13 cells). The procedure followed was based on previously described methods [25,27]. Cells were incubated with a fixed concentration of MAL (1 mM) and, thereafter, were exposed to different red light doses to causing survival rates of 5-10 %. The surviving cells were harvested 24 h after PDT and re-plated, allowing them to grow and then subjecting them to a new PDT treatment. The final population had received 10 PDT cycles and two resistant populations were selected: one subjected to five PDT cycles and the other exposed to 10 PDT cycles (SCC-5G and SCC-10G, respectively). The resistance for each population was checked by the MTT assay, indicating that the PDT conditions required to obtain SCC-5G and SCC-10G were more intense than that for SCC-1G. SCC-5G and SCC-10G showed an 8.5 and 9.5 increase fold in resistance to PDT (MAL 1 mM and 7.31 J/cm^2 red light dose), respectively, compared to SCC-1G and parental cells.

Similarly, PDT-derived variants were established after five consecutive ALA-PDT treatments, by using three different cell types, lung adenocarcinoma (CL1-5), breast carcinoma (MDA-MB-231) and melanoma (A375) cells [32]. However, in this case, the authors indicated that the obtained populations did not show relevant resistant properties.

Recently, Kim et al. [60] have isolated PDT-resistant variants from human oral cancer cells (Fadu) by repeated PDT treatments, up to 15 cycles. They exposed the cells for 24 h to 1 μM or 2 μM of hematoporphyrin following 15 min of irradiation (4.5 mW/cm^2). Surviving cells were recovered for 24 h. Then, the PDT-treated cells were again exposed to PDT. They checked the expression of proteins related to cell survival in PDT resistance cells, PARP-1 and LC3, by western blot; as well as the cell survival, by MTT. The authors indicate that the acquired PDT resistance would be due to LC3II and PARP-1 overexpression, and conclude that PDT resistance is related to autophagy by PARP-1 regulation in oral cancer cells.

In addition, some studies have been performed employing cells that highly express defined resistance markers. For instance, the ATP-dependent transporter ABCG2 is expressed at different levels in many cell lines used for in vitro and in vivo models for PDT, and it may affect their phototoxic efficacy [40]. Yu and Yu [41] treated primary cultures from a head and neck (HNC) tumor with ALA-PDT and they studied the photosensitizing effect on CSCs markers, particularly on ALDH1. They observed that ALA-PDT treatment effectively reduced CSC-like properties, including ALDH1 activity, CD44, Oct4 and Nanog positivity, self-renewal and invasion. Also Bostad et al. [39] selected cells with CSC markers; they used CD133 as CSC marker and isolated positive cells (CD133^{high} WiDr cancer cells of colon) by fluorescence

activated cell sorting. This population had a 7-fold higher capacity to initiate spheroids than $CD133^{low}$ cells and was more resistant to PDT. Furthermore, recently, Wei et al., [61] have isolated CSCs from colorectal cancer cells (primary cultured cells from patients and HT29 cell line) using a surface marker commonly found on stem cells of various tissues, PROM1/CD133 (prominin 1). They demonstrated that PpIX-mediated PDT induced the formation of autophagosomes in cells that express such marker accompanied by the upregulation of autophagy-related proteins. They conclude that there is a protective role played by autophagy against PDT in CSCs being of interest in order to develop novel therapeutic approaches for cancer. All these studies indicate that various CSC markers can be evaluated and related with the response to PDT.

Finally, it is important to emphasize that the level of resistance observed is lower in PDT-resistant variants than that reported for most drug-resistant cell lines. This could be due, among other things, to the specificity of the treatment. Chemotherapeutic drugs are quite specific and there are usually a single or a few subcellular targets (DNA, enzyme, receptors), acting directly on a membrane-bound glycoprotein transport system, or on specific target and then altering cellular pathways. However, there are numerous sites and types of injury associated with PDT, and overlapping mechanisms are therefore involved in PDT-cytotoxicity and resistance. Accordingly, it would not be expected to produce the same degree of resistance as it is observed with chemotherapeutic drugs if the sensitivity of cellular PDT targets or repair systems is modified. Therefore, the levels of resistance over a 1.5-fold increase in survival at the LD90 or LD50 are considered suitable in the generation of PDT-resistant cells. In addition, the relative resistance to PDT for the tumor cell lines is photosensitizer-specific.

In vivo Resistance Selection Models

One of the advantages of the use of *in vivo* models is that they provide us a tumoral micro-environment, being more "real" than *in vitro* ones. In this sense, cancer cells with intrinsic resistance and/or resistance acquired can be injected in immunodeficient mice. There is a wide number of published papers based on the use of this strategy and related with the resistance to chemotherapeutic drugs [15,16]. However, not many studies have been performed to obtain cells resistant to PDT [7,25-27].

The most frequently used *in vivo* models are mouse tumor allograft system (also known as syngeneic model), obtained by inoculating mouse cancer cells in mice; human tumor xenografts, resulting from the inoculation of human cancer cells in mice; and cell lines obtained from tumors induced by chemical or physical carcinogens (e.g. arsenic acid and ultraviolet/ionizing radiation, respectively).

The isolation of PDT resistant cells in mice can be carried out through two main selection strategies (Figure 2): (i) by using an original cancer cell population obtained from a primary or established culture; or (ii) by using a resistant cell population obtained from an original culture subjected to repeated PDT treatments or from cells with determined molecular markers.

The inoculation of the cancer cells is performed by subcutaneous injection into the dorsal flank, although they can be also orthotopically implanted into the organ of origin. Following the injection, tumor cells become palpable and can receive repeated PDT treatments with the aim of eradicating the tumor. After a variable period in which cells are subjected to successive treatments, if resistance occurs, the surviving tumor cells continue to proliferate, so they can be explanted and cultured for cellular and molecular resistance studies. With either subcutaneous

injection or orthotopic implantation, the tumoral environment within the host will contribute to the selection of those cells with resistant characteristics (Figure 2).

There are not many *in vivo* studies related to the selection of PDT-resistant cells. One of them was performed by Adams et al. [62]. They analyzed the response to PDT mediated by Photofrin in tumors derived from two cell types which showed in vitro resistance to PDT: RIF-1 and RIF-8A mouse fibrosarcoma cells. The authors found that the photodynamic treatment induced a significant reduction in the volume of both RIF-1 and RIF-8A tumors. However, after PDT, the re-growth was significantly slower for RIF-1 compared to RIF-8A tumors. In addition to this publication, the most of the in vivo research carried out in mice inoculated with cancer cells, has been focused on determining the efficacy of PDT with different PSs. In this respect, multiple cancer cell lines and numerous PSs have been evaluated. Some recent examples are: 4T1 murine mammary cells, using HPPH as PS [40]; HCT116 human colorectal carcinoma cells and DH-II-24-mediated PDT [63]; MCF-7 mammary cells and pheophorbide a as PS [64]; non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) treated with chlorin e6 – polyvinylpyrrolidone-mediated PDT [65]. Apart from this objective, mice models have been used to evaluate the role of determined molecular markers in PDT response. Thus, Tang et al. [66] analyzed the therapeutic potential of PDT pheophorbide a-mediated PDT in the multidrug resistance (MDR) of R-HepG2 human hepatoma cell line.

A first approach to characterize PDT-resistant Cells

Following the isolation of cells which resist to the photodynamic treatment, it is convenient to carry out their characterization, comparing them with the parental population. The resistance fold change would be determined *in vitro* using the classical viability assays such as the MTT

colorimetric assay or clonogenic assays [20-23]. Also, resistance fold change could be established *in vivo* through the evaluation of the tumor size reduction [67]. In addition, other aspects such as structural changes or biochemical, molecular and/or functional differences between the parental and the PDT-resistant populations, would be important for a better evaluation of PDT-resistance and to elucidate the potential altered mechanisms associated with susceptibility to PDT. Here, we just enunciate some of the aspects that could be evaluated.

Cell Morphology and Population Characteristics

Regarding to cell morphology, the results obtained by different laboratories indicate that it is altered in isolated PDT-resistant cells compared to the parental population. For instance, RIF-1 cells subjected to Photofrin II-mediated PDT showed an increase in cellular and nuclear size in comparison to the parental cells [25,31]. They also showed a total cellular protein content significantly higher than the parental cells. Concerning the plating efficiency, the results obtained depend on the isolation conditions. Similar results were described for the PDT-resistant Clon 4 and Clon 8 cells (isolated from LM3 cell line). In terms of protein content, it was higher in the resistant clones than in the parental cells (2-fold increase) [27]. Nevertheless, the plating efficiency was significantly impaired (25–30%) in both Clon 4 and Clon 8 compared to LM3, as well as the growth rate [68]. In the case of SCC-13 cells and their PDT-resistant variants, there were no substantial differences on the cell size, the plating efficiency and the distribution of the cells through the cell cycle (unpublished results from our laboratory). SCC-13 cells present variable morphological characteristics and may exhibit polyhedral shapes or fibroblastoid morphology with long prolongations [33]. This was also observed in the resistant isolated generations, though they showed a higher proportion of fibroblastic forms with respect to the

parental population. In the same way, it has been described that isolated Clon 4 and Clon 8 cells exhibit a more fibroblastic, dendritic pattern, and a higher spreading than LM3 parental line [68].

A higher degree of nuclear heterogeneity is generally present in cultured PDT-resistant cells. Moreover, long nuclear connections can be found between nuclei of mitotic cells and giant nuclei (polyploidy) are also observed in higher proportion in resistant cells than in parental cells [25,33,69]. Also, it has been widely published that resistant populations show an increase in the number of cells with micronuclei. This has been described for many types of resistant cells, such as the hepatocellular carcinoma HepG2 cell line (subjected to etoposide treatment) [70], the human endometrial adenocarcinoma HEC-1 cell line (treated with paclitaxel) [71] or SCC-13 PDT-resistant variants [33].

Furthermore, by using a comparative genomic hybridization array (aCGH), Gilaberte et al. [7] reported that both PDT-resistant and parental SCC-13 cells presented amplicons in the 3p12.1 CADM2, 7p11.2 EFGR and 11q13.3 CCND1 genes, but the resistant cells showed a distinctive amplicon in 5q11.2 MAP3K1, which was not present in the parental cells. These changes, confirmed by western blot, suggest that genomic imbalances related to CCND1, EFGR and, particularly, MAP3K1, could be implicated in the development of SCC resistance to PDT. Previous studies indicated that PDT can produce single and double strand breaks, sister chromatids exchanges, chromosome aberrations and mutagenic alterations [72,73]. This supports the results described in the resistant populations and indicates that such alterations could be related with the resistance process, as it has been also described in different resistant tumors treated with diverse chemotherapeutic agents [74-76].

With respect to the subcellular localization of the PSs and its intracellular accumulation, and taking altogether the published data, it is not clear that differences between parental and resistant cells are would be the cause of the differential response to PDT after identical treatment conditions. Several reports indicate the importance of the ATP-binding cassette transporter protein (ABCG2) in the regulation of PSs transport in different cell lines and its role in the response to PDT [50,51,77]. Thus, many studies have to be performed to better determine the importance of intracellular accumulation of the PS in the response to PDT in resistant cells.

Overexpression or mutations in specific growth factors, growth factor receptors and signal transduction proteins lead to a sustained signaling of proliferation and survival, as well as an aberrant proliferation, which contribute to PDT-resistance. Some examples of alterations in molecular markers that could be tested are: (i) ‘‘gain-of-function’’ gene alterations, such as the PI3K/Akt/mTOR and MAPK/ERK pathways [78-80]; (ii) inactivating mutations in tumor suppressor genes, such as RB (retinoblastoma), PTEN (phosphatase and tensin homologue deleted from chromosome 10) and P53 [81,82]; (iii) alterations in the machinery of apoptosis or autophagy, including overexpression of IAPs (anti-apoptotic proteins), like Bcl-2 or survivin and inactivation of pro-apoptotic genes, such as genes encoding caspases or proapoptotic Bcl-2 members [83-85]; (iv) oxidative and stress genes and proteins, such as HO (hemeoxigenase), HSP (heat shock proteins), SOD (superoxide dismutase) or glutathione peroxidase [86,87]; and (v) proteins related with ATP-binding cassette transporter [40,50,51].

Tumor Induction and Metastatic Abilities in Mice

There are different animal models to study drug resistance and each of them involves advantages and disadvantages, as reviewed by Rottenberg and Jonkers [88] and Politi and Pao [89]. Usually,

tumor cells are injected into immunodeficient mice for testing the tumorigenicity and the metastatic abilities of cultured cancer cells. This strategy is used by the researchers to test the features of PDT-resistant cells. Thereby, Luna and Gomer [25] analyzed the tumorigenic ability of the two PDT-resistant variants of RIF-1 mouse tumor cell line, obtained by repeated treatment with PII-mediated PDT. According with the obtained results, the number of cells required to produce palpable tumors in 50% of inoculated mice (known as latency time) was higher for resistant variants than for parental population. Similar results were obtained when athymic "nude" mice were used as host animals. Moreover, alike results were found by Casas et al. [68] when evaluating the ability of the parental LM3 cells and their PDT-resistant clones (Clon 4 and Clon 8) to grow subcutaneously in mice in order to form primary tumors and spontaneously to metastasize to the lung. The authors found that the percentage of mice that developed palpable tumors at latency time was lower in the resistant clones compared with the LM3 line, more markedly in Clon 8. They also evaluated the spontaneous lung metastasis induced by LM3 and resistant clones. Whereas LM3 cells metastasized to the lung in a tumor-size dependent way, Clons 8 and 4 almost did not induce nearly any metastasis at all. They linked these results to the impaired changes in cell adhesion found in the resistant clones compared with parental LM3 cells. The conclusion of both studies indicated that the ability of the PDT-resistant cells to induce tumors is lower than that of the parental ones.

These results contrast with those recently published by our laboratory [7], in which the behavior of the SCC-13 parental cells and their PDT-resistant populations was different (Figure 3). Both the parental and the resistant cells formed progressively growing tumors. However, the tumors induced by the PDT-resistant cells were bigger than those induced by the parental cells. The histological characteristics of the tumors were also different; whereas the tumors induced by

parental cells were well or moderately differentiated squamous cell carcinomas, those induced by PDT-resistant cells were mostly moderately or poorly-differentiated SCCs, formed by atypical keratinocytes with nuclear pleomorphism even infiltrating skeletal muscle fibers. The metastatic abilities of the resistant SCC-13 cells were not evaluated. Although it has not being performed with PDT-resistant cells, previous results published by Momma et al., [90] using an orthotopic prostate cancer obtained by inoculation of the MatLyLu variant of the Dunning 3327 rat prostate cancer cell line, treated with benzoporphyrin derivative, showed that PDT produced a significant increase in the mean number of lung metastases. The authors indicated that different factors may need to be evaluated when considering PDT for primary prostate cancer.

It should be noted that the observed differences in the ability to induce tumors between PDT-resistant cells obtained in the different studies could be due to a variable number of factors. In the studies carried out by Luna and Gomer [25] and by Casas et al., [27] resistant clones were isolated; whereas in our case, a resistant population was selected. As it has been indicated before, it is possible that, by using the cloning methodology, the optimal resistant clones to study tumorigenicity in mice are not necessarily selected, since in the resistant cell population cells with different tumorigenic abilities are present. Obviously, other factors may contribute to the dissimilarities obtained, including the cell line, the PS employed and the treatment conditions.

Conclusion

The resistance to anticancer therapies constitutes a relevant unsolved problem in the treatment of cancer. One of the current goals in PDT lies in modelling, in cellular and animal systems, the characteristics associated to tumor resistance. The assays based on cancer cell cultures and mice models are excellent tools available to diagnose both the intrinsic and the acquired resistance,

which may develop rapidly as the result of repeated treatment cycles. The behavior of PDT-resistant cells depends on different factors, including the cell line, the PS or prodrug used and the way of selecting them. Nonetheless, these cellular models may reveal useful information about the molecular basis of intrinsic and acquired resistance to PDT.

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Figure Legends

Figure 1. Different ways to obtain *in vitro* PDT-resistant cells from a cell culture. The whole population is subjected to repeated treatments and a small percentage of heterogeneous cells can resist due to the development of resistant characteristics (a); selection of clones with determined mutations and new mutations developed after repeated PDT-treatments (PDT would positively select first those cells that present intrinsic resistant mechanisms) (b); cells expressing determined molecular markers are selected after PDT-treatments (c).

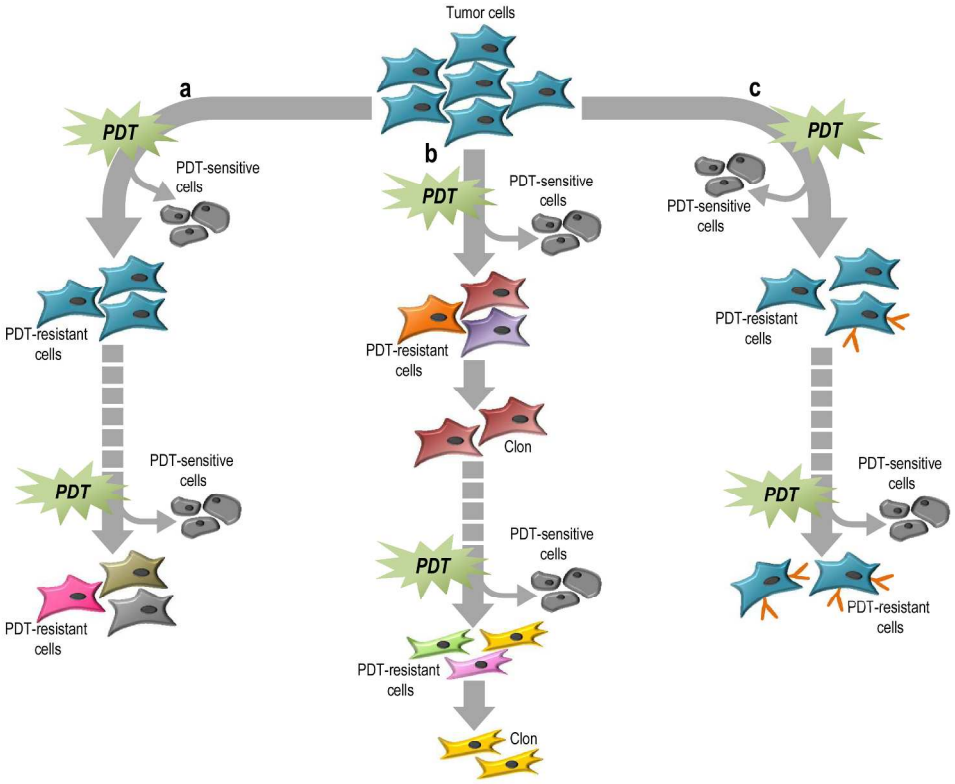
Figure 2. Two possible ways to select *in vivo* PDT-resistant cell populations. In the first way, a resistant cell population obtained from a primary or established culture is subjected to repeated PDT-treatments and then injected into mice. In the second way, an original cancer cell population is injected into mice and when the tumor becomes palpable, receives repeated PDT-treatments. If resistance occurs, the surviving tumor cells continue to proliferate. In both cases, resistant cells can be explanted and cultured for cellular and molecular studies.

Figure 3. Tumor development after inoculation of parental squamous carcinoma cells (SCC-13) or their PDT-resistant variant in immunosuppressed mice. Parental and PDT-resistant SCC-13 cells were injected in the right and in the left flanks of the mice, respectively. The tumor induced by the resistant variant was bigger than the one derived from the parental line. Then, histopathological analysis revealed that the tumor induced by the resistant variant presented characteristics of moderately or poorly differentiated squamous cell carcinoma. In addition, squamous cells infiltrating the skeletal muscle were observed. However, the tumor induced by the parental cells showed characteristics of well differentiated squamous cell carcinoma with dyskeratotic cells and keratin accumulations. These differences were also seen

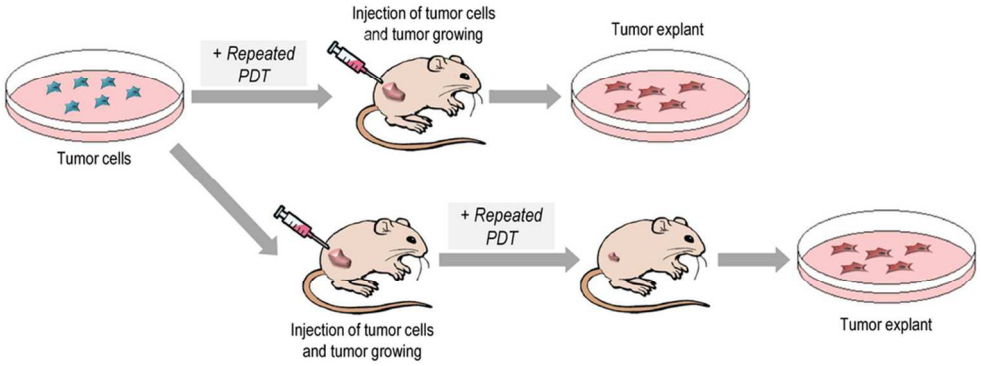
by using immunostaining against involucrin protein to highlight human cells forming the xenograft tumor.

Cell type	Origin		Treatment conditions			Nº of cycles	Type of selection	Resistance fold change	Reference
	Specie	*Cancer type*	*PS and concentration*	*Incubation time*	*Light dose (J/cm²)*				
RIF-1	Mouse	Radiation-induced fibrosarcoma	Porphyrin (25 µg/mL)	1 h	0,2 - 0,84	10	Clonogenic	1,2 - 1,5 log units	Luna et al. 1991
				16 h				2,5 - 3 log units	
RIF-1	Mouse	Radiation-induced fibrosarcoma	Zinc (II) pyridinium-substituted phthalocyanine (0,98 µM-9,8 µM)	1 h	3	15	Total population	7,1	Mayhew et al. 2001
			Polyhaematoporphyrin (2 µg/mL – 25 µg/mL)					5,7	
HT29	Human	Colon adenocarcinoma	Photofrin (1 - 100 µg/mL)	18 - 4 h	2,7	1 - 14	Clonogenic	1,5	Singh et al. 2001
			Nile Blue A (1 - 3µg/mL)	1 h - 30 min	8,1	1 - 8		2,62	
			Aluminum phthalocyanine tetrasulfonate (25 - 60 µg/mL)	18-4 h	5,4	1 - 11		2,16	
LM3	Mouse	Mammary adenocarcinoma	ALA (0,6 mM)	3 h	0,36 - 5,4	13	Clonogenic	6,7 (Cl4) and 4,2 (Cl8)	Casas et al. 2006
CL1-5	Human	Lung adenocarcinoma	ALA (1 mM)	3 h	3 - 18	5	Total population	-	Tsai et al. 2009
A375		Melanoma			1 - 9				
MDA-MB-231		Breast carcinoma			1 - 6				
SCC-13	Human	Squamous carcinoma	Me-ALA (1 mM)	4 h	7,31 - 25	10	Total population	8,5 - 9,5	Milla et al. 2011
Fadu	Human	Pharynx squamous carcinoma	Hematoporphyrin (1 µM or 2 µM)	24 h	4,5	15	Total population	12,25 - 7,75*	Kim et al. 2014

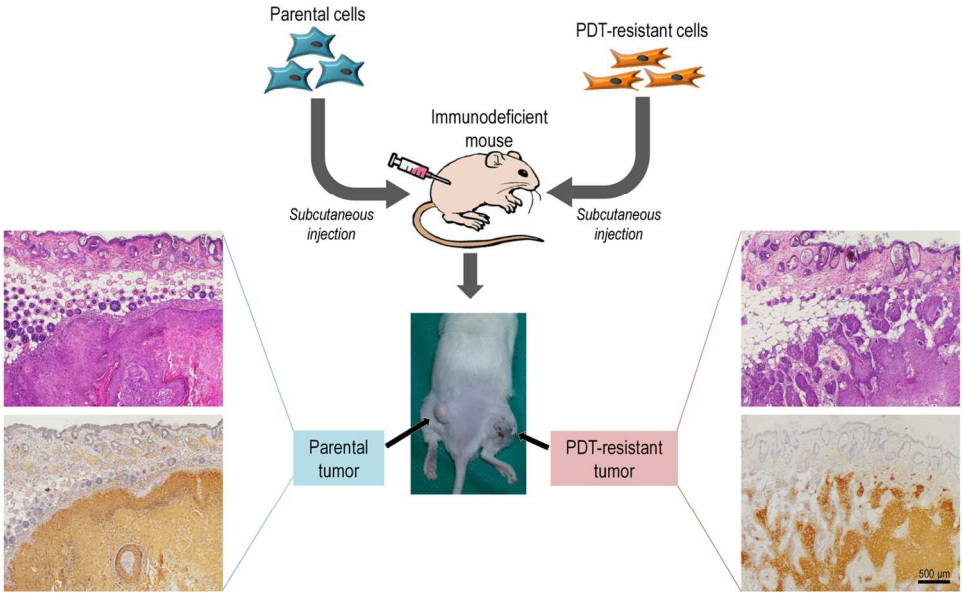
*These data approximately correspond to a 4 µM HP-PDT treatment to 15 cycles-resistant cells.



235x194mm (300 x 300 DPI)



89x36mm (300 x 300 DPI)



137x86mm (300 x 300 DPI)