

Chapter 5

Isolation and Initial Characterization of Resistant Cells to Photodynamic Therapy

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Abstract After photodynamic therapy (PDT), the apparition of resistant tumor cells can occur. Laboratory models are being developed in order to understand the potential mechanisms implicated in such resistance. In this sense, we describe the methods published for the isolation and characterization of tumor cells resistant to PDT. We also propose other unpublished procedures that could be of interest for the study of cells resistant to PDT. Factors such as the parental cell line, the photosensitizer (PS) (or prodrug), the photodynamic treatment conditions, the treatment interval, and the clonal or total population selection have to be taken into consideration. Treatment doses are generally high and repeated over time. The development of resistant cells to PDT could take several months. The characterization of resistant cell populations vs parental cells can be performed by using different cellular and molecular techniques, including: cell morphology analysis, intracellular PS content measurement, PS localization, migration and invasion capacity, expression and distribution of adhesion proteins, death proteins and evaluation of specific genes implicated in cell proliferation and survival. Transplantation mouse models also contribute to determine the biological activity of the PDT-resistant cells *in vivo*, allowing the evaluation of their tumorigenicity and aggressiveness. Laboratory cell models will help us to understand how resistance to anticancer PDT affects the biological and functional aspects of tumorigenicity *in vitro* and *in vivo*, which are necessary to improve the clinical results.

Keywords Isolation · PDT · Photodynamic therapy · Photosensitizer · Resistance · Tumor cells

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V. Rapozzi, G. Jori (eds.), *Resistance to Photodynamic Therapy in Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 5, DOI 10.1007/978-3-319-12730-9_5

Abbreviations

ALA	δ -aminolevulinic acid
ALDH1	aldehyde dehydrogenase 1
BCC	basocellular 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-hexyloxyethyl)-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

Introduction

Resistance to anti-cancer therapies is the main cause of their failure, leading to tumor progression and poor clinical prognoses. Thus, a deeper understanding of how resistance affects the biological and functional aspects of tumorigenicity is necessary to enhance the efficacy of cancer treatments. Resistance to chemotherapy as well as radiotherapy has been broadly studied; however, the process is far from being well understood. The effectiveness of the treatment for specific cancers is limited by drug resistance and, in the same way, recurrence after radiotherapy continues to pose a major obstacle [1–5]. Although it is not well documented, Photodynamic Therapy (PDT) of cancer can also induce tumor cell resistance in patients [6–9]. Therefore, the development of cellular and/or animal models, based on the selection of resistant cells, that allow a better understanding of this process, is an important goal in the research of the types of cancer in which this therapeutic modality is being applied, including cancers of the head, neck, lung, esophagus, urinary bladder, gynecological cancers and particularly, non-melanoma skin cancer (NMSC)

[10–15]. The complexity of genetic and epigenetic alterations of tumors invariably highlights complex situations, but the development of this kind of models will be very useful to perform systematic, molecular and functional studies, to analyze the mechanisms underlying PDT-resistance.

In cancer therapy, the first treatment usually kills most tumor cells; however, some tumors do not react properly to the therapy and resistant cancer cells can become even more aggressive after several cycles of treatment. In general, resistance can be classified into two types: (i) intrinsic, in which resistance-mediating factors pre-exist in the tumor cells before receiving treatment, and (ii) acquired, which is developed as a consequence of the treatment in tumors initially sensitive. Intrinsic resistance arises from a complex range of biochemical and molecular characteristics of the tumor which result in the cells death escape. Acquired resistance can be caused by different factors, including the limited amount of drug or radiation reaching the tumor, those affecting the tumor micro-environment, as well as mutations in tumor cells arising during treatment [16–19]. Other adaptive responses, such as increased expression of the therapeutic target and activation of alternative compensatory signaling pathways, have to be also considered. Furthermore, it is recognized that tumors can contain a high degree of molecular heterogeneity with genotypic or phenotypic variations [20–22]. This intratumoral heterogeneity implies that different parts of a tumor may have different properties, apart from the existence of different degrees of sensitivity to different treatments. Furthermore, the heterogeneity can lead to variations in the specific mechanisms of response induced by the therapy. In addition, in the acquired resistance, the tumors not only become resistant to a particular therapy originally used to treat them, moreover they may develop cross-resistance to other therapies with different mechanisms. This is particularly evident in chemotherapy, where tumor cells can become resistant to multiple drugs. Therefore, resistance can arise through therapy-induced selection of a cell population that developed resistant characteristics and/or from a resistant minor subpopulation of cells present in the original tumor with determined characteristics.

PDT can leave a significant number of surviving tumor cells which have been exposed to reactive oxygen species arising when the photosensitizer is excited by light, but insufficient to destroy them. Potential changes in the rates of cell division, death, mutation or migration would have direct effects on the tumor growth and also in the response to a new photodynamic treatment with biological consequences. In this context, we describe different methods to isolate tumor cells resistant to PDT as well as some of the resistant characteristics, which facilitate a better understanding of the mechanism of action of PDT to enhance its efficacy.

Isolation of PDT-Resistant Cells

A better understanding of the *in vitro/in vivo* characteristics of PDT-resistant cells allow us to study the long-term molecular, biochemical and cellular changes induced by the treatment. This can be exploited to selectively treat the surviving cells

with modified PDT protocols or with other therapies. Some drug-resistant cell lines for chemotherapeutic agents as methotrexate, vinblastine or terephthalanilide were first developed around 1960 by using *in vivo* mouse models [23]. The *in vitro* development of resistant cancer cell lines was early described in chemotherapy in 1970 [24]. The research isolated resistant cell lines from chinese hamster cells using an increased treatment dose with actinomycin D. The cells showed a 2500-fold greater resistance to the drug than parental cells, and these resistant cell lines were also cross-resistant to other chemotherapy drugs, such as vinblastine and doxorubicin. Many examples of drug-resistant cells isolation have been reported since then in the literature. In addition, isolation of resistant cells to other cancer therapies has also been described. Hahn and van Kersen [25], for instance, obtained heat-resistant cell strains from mouse radiation induced fibrosarcoma cells (RIF-1) by repeated heating (11 heating and regrowth cycles) of cells derived from survivors of previous heating cycles (60 min; 45 °C). They selected several thermally resistant strains derived from single cells that had survived. The resistant cells showed a growth rate and plating efficiency similar to that of RIF-1 cells and no obvious morphological abnormalities were described. In the case of PDT, resistant cells have been obtained by using exogenous PSs, such as Photofrin and phthalocyanines among others, and with the endogenous PS protoporphyrin IX (PpIX), formed from δ -aminolevulinic acid (ALA) or methyl δ -aminolevulinic acid (MAL) through the heme biosynthetic pathway [26–28].

Resistance against anticancer therapies has hardly been studied in animal models until very recently. The *in vivo* models provide the native microenvironment in which tumors reside, being, therefore, more “real” than the *in vitro* ones. However, although there are a wide number of published papers in chemotherapy [18–19], no much work has been performed to evaluate resistance to PDT [8, 26–28]. The most frequently used *in vivo* models are the mouse tumor allografts (or syngenic) and the human tumor xenografts, obtained by inoculating both immortalized mouse or human cancer cells, respectively. Small fragments from tumors showing intrinsic resistance to anticancer agents can also be injected. In addition, and to avoid the rejection of the implanted cancer cells, mice used for allografts or xenografts had impaired immune systems. Tumors induced by chemical or physical carcinogens (ultraviolet or ionizing radiation) can also be used to obtain cell lines, with innate or acquired resistance, due to a determined therapy.

Defining the Level of Resistance

In chemotherapy, drug-resistant cell models are generated in the laboratory, principally by repetitive exposures of culture cancer cells to increased concentrations of drugs. The surviving resistant cells are then compared to the parental sensitive ones using different assays (e.g. viability/proliferation assays, such as the MTT, or the clonogenic assay) [23, 29, 30]. Similar strategies have been used to generate PDT-resistant cells using repeated photodynamic treatments as it will be described later.

Cellular sensitivity is determined by exposing them to previously defined treatment conditions followed by assessing cell viability to achieve the Fold Resistance Index. In chemotherapy, the drug concentration that causes 50% growth inhibition (IC50) is the main index used to determine the increase in resistance (Fold Resistance $IC_{50} = \text{Resistant Cell Line}/IC_{50}$ of Parental Cell Line). In PDT, there is not a well-defined way to describe it. The Fold Resistance Index would refer to selected treatment conditions (PS concentration and dose light irradiation) to induce a lethality of 50% (LD50) or 90% (LD90) in the parental cell line (Fold resistance = Resistant Cell Line/Parental Cell Line).

To establish the level of drug resistance that occurs in the clinical treatment of cancer, the ideal situation would be to compare cell cultures established directly from cancer patients before and after chemotherapy. Data from chemotherapy have been recently summarized by McDermott et al. [23], indicating that the majority of cell lines developed after chemotherapy from patients with lung, neuroblastoma or ovarian cancers, showed from 2–5-fold increase in resistance to the agents from the IC50 value of the parent cell line. The fold increase in PDT-resistant vs parental cells, in general, is not so high, but it is considered as resistant variant a 1.5-fold increase over the parental cells.

Different models for PDT can be developed but, in general, once it has been selected the treatment conditions of drug concentration and light radiation dose to induce a LD50 or LD90, tumor cells are subjected to repeated PDTs and total resistant populations or specific resistant clones are selected from the mixed population.

The main objectives are to develop an *in vitro* model where repeated therapy is extensively used to achieve large fold resistance vs parental cells, and to obtain a stable phenotype in the resistant cells. Some factors have to be taken into account to create the model including the parental cell line and the treatment conditions (drug concentration and light radiation dose) used, that must be optimized depending on the parental cell line selected for use in developing the resistant model. The recovery rate after the treatment is also important since there can be differences between PSs, even at equivalently cytotoxic treatment conditions administered to cells. After the treatment with the selected dose, cells must be able to return to logarithmic growth, ensuring the selection of resistant cell subpopulations.

Selecting the Cellular Model

In cancer research, the study of the cellular and molecular bases of intrinsic and acquired resistance to cancer therapies, including PDT, could be performed by using mainly two types of *in vitro* models: (i) primary cell cultures, directly obtained from human or mouse tumors, and whose sensitivity or resistance to the anticancer therapy has to be later evaluated, and (ii) immortalized cancer cell lines, showing or not primary resistance.

In the first option, the ideal situation would be to select a chemotherapy and radiation untreated cell line since previous treatment may have already caused changes

in the resistance pathways and increased the expression of drug resistance markers that may not be relevant to the therapy being studied. However, cell lines derived from untreated human tumors are relatively rare and most of the cells used in these studies are been derived from treated tumors. In relation to immortalized cancer cells, many cell lines are available for each cancer type, carrying different genetic alterations to choose the most suitable *in vitro* models to investigate mechanisms of resistance to PDT. In the case of chemotherapy, for instance, there are two important sources to 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 molecular markers of drug response; and the Cancer Cell Line Encyclopedia (CCLE, www.broadinstitute.org/ccle) which includes data related with gene expression or chromosomal copy number [31, 32]. Therefore, there are numerous cell lines available and the selection will depend on the kind of study to carry out. In the case of PDT, not much research related to PDT-resistant cell selection has been performed, and those which have been carried out have used immortalized cell lines, including the mouse radiation-induced fibrosarcoma (RIF-1) cells [26, 33]; murine mammary adenocarcinoma (LM3) cells [28]; human colon adenocarcinoma (HT29) cells [34]; human lung adenocarcinoma (CL-5) cells; human melanoma (A435) cells and human breast carcinoma (MDA-MB-231) cells [35]; and human squamous cell carcinoma SCC-13 cells [8, 36].

Strategies for Selection of Resistant Cells

A validated protocol to reproduce resistance under the therapy-induced pressure in preclinical studies is based on an *in vivo/in vitro* selection of cancer cells with intrinsic or acquired resistance after chronic treatment. In any case, the scenarios to select resistant cells to PDT are multiple, and here we describe some of many different possibilities.

In Vitro Selection

In the *in vitro* system, apart from the cells selected (primary or established cell line) for the isolation of the resistant cells, it should be taken into account that tumors are heterogeneous and, then, their different subpopulations have different properties. Consequently, the cancer cell lines derived from them would be also heterogeneous [20–23]. In any case, and once the cell line has been chosen, there are three basic selection strategies for isolating anticancer therapy resistant cells: (1) selecting a small resistant population from the original culture; (2) selecting resistant clones and (3) selecting cells with determined molecular markers (Fig. 5.1a).

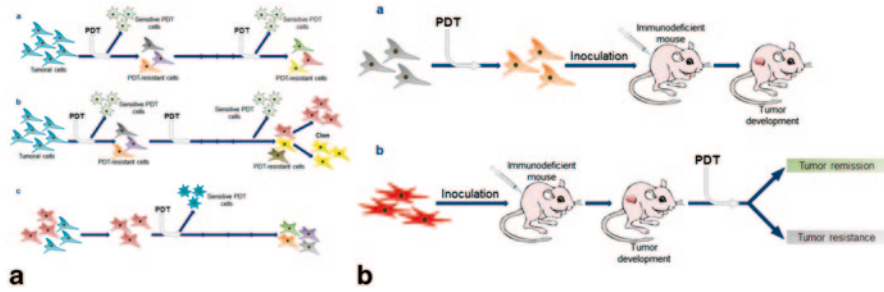


Fig. 5.1 Different ways to obtain *in vitro* (a) and *in vivo* (b) PDT-resistant cell populations. **a** The whole population is subjected to repeated treatments and a small percentage of potential heterogeneous cells can resist due to the development of mutations (a); cell clones with mutations and with new mutations developed after repeated PDT-treatments are selected. PDT would positively select first those cells that possess intrinsic resistant mechanisms (b); selecting cells expressing determined molecular markers and, then, expose them to PDT-treatments (c), **b** heterogeneous cell population is injected in the mice and then subjected to repeated PDT-treatments (a); cells are first subjected to repeated treatments and the resistant cell populations are inoculated in the mice (b)

1. Selecting a Resistant Population from the Original Culture

Basically, when a punctual treatment is given to a cell population, a small percentage of cells can resist, being responsible of the repopulation of the culture. This resistant cell population is again exposed to a new treatment and most probably the selected cells would be heterogeneous and differ from the original parental cells, due to the apparition of genetic or epigenetic alterations that promote their survival [23, 37, 38]. In this sense, heterogeneity has been seen in taxane-resistant models developed from human lung cancer cell lines [29, 37, 38] or in human breast cancer cells [30]. There is also another possibility, that the selection results in the isolation of a cell population that already had a resistant signature in the original culture. Indeed, this has been demonstrated for many drug-resistant models, which are often enriched with markers of cancer stem cells (CSCs). CSCs are thought to be responsible for tumor regeneration after chemotherapy and radiotherapy and they would also have a role in resistance to PDT.

Therefore, it is tempting to consider that from a heterogeneous group of initial cells, PDT would positively select those cells that suffer determined genetic alterations or possess intrinsic resistant mechanisms, while cells that do not have such acquired alterations or intrinsic mechanisms die after treatment. In addition, the therapy would progressively stimulate a higher expression of molecules that induce resistance, including additional mutations. The resulting resistant variant cells could be also finally selected, for instance, by cell sorting using specific molecular cell markers.

2. Selecting Resistant Clones

The second method is selecting resistant clones by limited dilution. Clonal selection has the advantage that isolated cells would be more resistant to the treatment than others within the same cell line [23, 39, 40]. However, it must be taking into

account that such clones are not necessarily the responsible for the tumor relapse and, eventually, for metastasis. For this method of isolation, two possible strategies can be followed. The first option consists on selecting resistant clones after treatment, subjecting such clones to a second therapy/drug and again selecting the most resistant clones that can be again subjected to new treatments. This was the protocol used, for instance, in the isolation of colchicine resistant cells from the carcinoma cell line KB3–1, treated with three stepwise increases drug treatment [39]. Clones were collected from each round of the selection strategy. The other option consists on selecting resistant clones after several rounds of treatments. This also would allow investigating 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 [40]. The clones showed morphologically distinct characteristics; one of them overexpressed the ABC efflux transporter P-glycoprotein, whereas the other clone did not. Similar heterogeneity has been also described in cisplatin-resistant models developed from a human pancreatic cancer cell line with a mutation in DNA repair protein BRCA2 [41].

3. *Selecting Cells with Determined Molecular Markers*

The third method for isolation resistant cells to cancer therapies is based on the expression of differential molecular markers in the tumor cells. Both intrinsic and acquired resistance to anticancer therapies result from numerous genetic and epigenetic changes, therefore, for an effective cell selection a combination of different markers, based on specific individual genotypic and phenotypic variations in the resistant that cells can be used. Taking into account that in chemotherapy cancer cell resistance occur at different levels, including activation of oncogenes and inhibition of tumoral suppressors, variations in drug influx/efflux or evasion from apoptosis, different markers could be employed to identify them [17, 42, 43]. In addition, stem cell characteristics are also important factors in the resistance process [1, 44]. In the case of PDT, all of these factors would be implicated in promoting resistance. Alterations in the expression of many different genes have been observed and, therefore, multiple signaling pathways are contributing to PDT resistance [12, 45–47].

A very important factor in drug resistance is mediated by proteins which belong to the ATP-binding cassette (ABC) transporter family, which increase drug efflux and, thus, reduce the intracellular drug concentration. Among these proteins, P-glycoprotein (MDR, Pgp or ABCB1), multidrug resistance protein 1 (MRP1 or ABCC1) and ABCG2 are the most frequently associated with multidrug resistance. These proteins are expressed at variable levels in cancerous cells [48]. Accordingly, ABCG2 is being used as an important marker for selecting cancer cells by flow cytometry and magnetic-associated cell sorting (MACS). ABCG2 can bind and efflux a wide range of structurally different classes of PS used preclinical and clinically, such as porphyrins and chlorins. It is expressed at different levels on cell lines used in many *in vitro* and *in vivo* tumor models for PDT which may affect phototoxic efficacy [49, 50]. Among the PSs that are substrates for ABCG2 they included Photochlor, Benzoporphyrin derivative monoacid ring A (BPD-MA, Verteporfin), Hypericin and Protoporphyrin IX (PpIX), after exogenous administration of ALA.

ABCG2 may reduce the intracellular levels of the substrate PS below the threshold for cell death in tumors treated with PDT, leaving resistant cells to repopulate the tumor [51–53].

Another important factor in resistance to drugs is the epidermal growth factor (EGFR). Alterations in the protein lead to sustained activation of the MAPK/ERK signal pathway in many human malignancies including skin, colorectal, ovarian, breast, and prostate cancers, and often correlates with the enhanced cellular proliferation and development of cancer metastasis [54, 55]. Therefore, it is an important potential factor in the resistance to PDT. In this sense, in general, in cells with a good response to PDT, down-regulation of EGFR has been noted in PDT-treated cells *in vitro* and *in vivo*, and it has been suggested that the decreased cell migration and the invasiveness in RIF-1-PDT-derived variants are related to the down-regulation of EGFR. Compared to parental CL1-5, A375 and MDA-MB-231 cells, ALA-PDT caused a reduction in the level of EGFR in PDT-derived variants, which correlated with the reduced migration and invasion in the PDT-derived variants [35]. However, it has been described that sustained ERK activation protected cells from PDT [56]. 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 [57]. It has been also demonstrated in patients with a bad response to PDT as well as in the resistant PDT-SCC-13 cells the up-regulation of EGFR [8].

Finally, several evidences suggest that tumors contain a small subpopulation of cells, the cancer stem cells (CSC), which exhibit self-renewal capacity, proliferate infrequently, express several pluripotency genes and are responsible for tumor maintenance and metastasis [44, 58, 59]. These slow cycling cells are not impacted/affected by anti-cancer agents that kill rapidly growing tumor cells, although 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 drugs resistant and relapse. In fact, in recent years, CSCs have been identified in several cancers and have been proposed to explain the metastatic capacity, recurrence, and resistance to radio therapy and chemotherapy [44, 60, 61].

Some markers have been associated to CSC. For instance, in breast cancer, the stem cell population is CD44+/CD24 and CD133 marks cancer stem cells in brain tumors, colorectal carcinoma and pancreatic carcinoma. In head and neck squamous cell carcinoma a CD44+ population of cells possesses the properties of CSC, and ABCG2 and aldehyde dehydrogenase 1 (ALDH1) activity have also been reported to identify cancer stem cells in a host of cancer types [62–64] and also for skin cancer [65, 66].

CSCs have been identified and isolated using different approaches including flow cytometry and magnetic-associated cell sorting. Therefore, recently, Adhikary et al. [67] selected a cell population from the squamous cell carcinoma SCC-13 and A-431 cell lines by using aldehyde dehydrogenase 1 as marker. Such isolated cells formed spheroids and induced larger tumors with faster growing in immunocompromised mice as compared to non-selected cells. Spheroid-selected cultures were highly enriched on the expression of epidermal stem cell and embryonic stem

cell markers, basically of aldehyde dehydrogenase 1 (ALDH1), keratin 15, CD200, oct4 and trimethylated histone H3, among others. These studies indicate that the subpopulation of cells that possess stem cell-like properties enhance tumor forming potential and can be selected by cell sorting using the human epidermal stem cell markers. These results are very interesting and aim to (i) evaluate the expression of stem cells in PDT resistant cells and (ii) to select firstly cells with CSC markers and test the sensitivity to PDT.

In vivo Selection

Likewise, as in the case of the resistant cell selection by using *in vitro* systems, cancer cells, with intrinsic and/or acquired resistance previously subjected to repeated treatments, may be injected in immunodeficient mice. These strategies are based in the studies performed on resistance to drugs in chemotherapy [18]. In this case, there would be two basic selection strategies for isolating PDT-resistance cells in mice by using: (1) a determined original cancer cell population or (2) a resistant population obtained from an original culture subjected to repeated PDT treatments or cells showing the determined molecular markers (Fig. 5.1b). Cancer cells will be injected in the mice subcutaneously (s.c.) into the dorsal flank, or orthotopically by implanting tumor cells into the organ of origin. After the injection, tumor cells become palpable and can receive repeated treatments with the selected compound/PSs to induce tumor destruction. However, after a variable period of continuous treatment, if resistance occurs, the “remnant” tumor cells proliferate again and the tumor cells can now be explanted and cultured for cellular and molecular resistance studies. In both cases, the tumoral environment in the host will contribute to select cells with resistant characteristics.

Not many *in vivo* studies have been performed for selecting PDT-resistant cells. Adams et al. [68] evaluated the response to *in vivo* PDT with Photofrin in tumors derived from RIF-1 mouse fibrosarcoma cells and in tumors derived from RIF-8A cells, which showed *in vitro* resistance to PDT. The authors found a significant reduction in the tumor volume similar for both RIF-1 and RIF-8A tumors, whereas the re-growth was significantly delayed for RIF-1 compared to RIF-8A tumors following PDT. They also evaluated the clonogenic survival of the cells obtained from explanted *in vitro* immediately following *in vivo* PDT treatment. Apart from this article, most of the studies performed in mice with different cell lines have been focused for determining the efficacy of PDT with different PSs. In this respect, many reports with different cell lines and PSs have been published. Some recent examples are: the murine mammary tumor 4T1 cells with HPPH as PS [50], the human colorectal carcinoma HCT116 cells with the chlorin-based photosensitizer DH-II-24 [69], the mammary MCF-7 cells and pheophorbide a [70], the non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) with chlorin e6– polyvinylpyrrolidone [71], among many others. In addition, these models have been used to evaluate the role of determined molecular markers in the PDT response. Hence, Tang et al. [72] studied the therapeutic potential of PDT in the multidrugresistance (MDR) human hepatoma cell line R-HepG2 with the photosensitizer pheophorbide a.

Examples of PDT Selection

In PDT, the generation of resistant cell variants will enable investigators to understand the molecular mechanisms of sensitivity to several photosensitizers, based on inherent and induced resistance in different cell lines. PDT-resistant cell lines have been obtained using various photosensitizers such as Photofrin, phthalocyanines or Nile Blue, as well as after exogenous incubation with precursors of PSs such ALA and MAL.

The first studies for the isolation of PDT-resistant cells were performed by Luna and Gomer [26]. They isolated PDT-resistant variants from the mouse radiation-induced fibrosarcoma (RIF-1) cell line, following a protocol of repeated porphyrin (Photofrin II, PII) incubation and light treatments. They used two incubation procedures, either an extended (16 h) or a short (1 h) incubation period to obtain resistant cells exposed to conditions with different intracellular photosensitizer localization. By cloning, they selected two individual colonies from each PDT porphyrin incubation time used. However, the morphological characteristics as well as the behavior of the different clones were different. All resistant variants had increased protein content and were larger than the parental RIF-1 cells. *In vitro* growth rates were similar. Flow cytometric analysis using propidium iodide showed the characteristic mixture of diploid and tetraploid subpopulations for the parental and one of the clones selected, whereas a complete tetraploid phenotype was present in the three other PDT-resistant variants.

Likewise, Singh et al. [27] induced resistant populations to PDT also from the RIF-1 tumor cells by repeated photodynamic treatment with PII (4 or 18 h of drug incubation) to the 0.1–1 % survival level, followed by regrowth from single surviving colonies. The resistance is shown as increased cell survival in the strain designated RIF-8A, compared to the wild-type RIF-1 cells, when exposed to increasing PII concentrations, 18 h of drug incubation and fixed light exposure. Resistance to PDT was also observed in Chinese hamster ovary-multidrug resistant (CHO-MDR) cells, compared to the CHO wild type cells by the same authors. These findings suggest that different mechanisms are responsible for PDT-induced resistance and multi-drug resistance. Lately, the same group, by using three different photosensitizers (aluminum phthalocyanine tetrasulfonate, AlPcS4; Nile Blue A and Photofrin), selected by their different localization properties, induced different resistant populations in three human cell lines: neuroblastoma (SK-N-MC), human colon adenocarcinoma (HT29) and human bladder carcinoma (HT1376) [34]. Cells were incubated for 1 h (Nile Blue) or 18 h (AlPcS4 and Photofrin) using two different drug concentrations and two different light doses. They evaluated the cell survival by the colony forming assay and the authors indicate that multiple cultures were performed from single surviving colonies. Cells were regrowth and treated again receiving between 8 and 14 cycles. Each treatment cycle was aimed at achieving survival levels in the 1–10% range, and they considered as PDT-resistant variants those cells with over 1.5-fold increase in PDT resistance. Resistant cells were isolated by the colony forming assay. Under such conditions, they obtained several resistant cell lines from HT29 using the three PSs and from HT1376 using the PS Nile Blue. However, 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 for different cell lines is not surprising. However, the different relative rankings with respect to resistance are very interesting and highlight the importance of the appropriate photosensitizer selection. Moreover, this could correlate with the understanding that the mechanisms and pathways of cellular death are sensitizer-specific. The authors suggest that a specific variation within the population or a selectively advantageous mutation during the repeated treatments facilitates the development of the resistant variants.

Using similar protocols, Casas et al. [28] isolated resistant clones of murine adenocarcinoma cells, LM3, after repeated ALA-PDT treatments. The authors used a fixed concentration, 0.6 mM, of the ALA pro-photosensitizer and varied the light doses (0.36–5.4 J/cm²) to achieve survival levels in the 5–10% range. The surviving cells were allowed to grow and were again subjected to a new cycle of ALA-PDT. The final population 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 to kill 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. [33], using polyhematoporphyrin (PHP) and Zn(II) pyridinium-substituted phthalocyanine (PPC) as PSs, isolated two RIF-1 resistant cell populations, and demonstrated a 5.7 and 7.1-fold increase in resistance, respectively. Both resistant strains were isolated following 15 cycles of photosensitization treatment with increasing sensitizer concentrations and fixed light doses. After the photosensitization cycles, the isolated strains were RIF-25R, from PHP treatment, and P10 strain, obtained after PPC treatment.

Milla et al. [36], using a cell line obtained from squamous cell carcinoma of skin (SCC-13 cells), developed resistance to PDT cells. The procedure followed was simple and based also as that previously described [26, 28]. Cells were incubated with a fixed concentration of MAL (1 mM) and, thereafter, exposed to different red light doses to cause survival rates of 5–10%. The surviving cells were harvested 24 h after PDT and replated, allowing them to grow and then subjecting them to a new PDT treatment. The final population received 10 PDT cycles and two populations were selected: one subjected to 5 PDT cycles and the other exposed to 10 PDT cycles (SCC-5G and SC-13–10G, respectively). The resistance for each population was checked by the MTT assay, indicating that the PDT conditions required to obtain the last SCC resistant generations were more intense, from 7.31 J/cm² to obtain the 1st to 25 J/cm² for the 10th generation. In addition, these resistant cells have a higher viability after PDT, compared to the parental cells. In fact, the parental and the 1st generation cells exposed to PDT (MAL 1 mM and 7.31 J/cm² red light dose) had a viability of 10%, while the 5th generation and the 10th generation had 85 and 95% of viability, respectively.

Likewise, by using three different cell types, lung adenocarcinoma (CL1-5), breast carcinoma (MDA-MB-231) and melanoma (A375) cells, PDT-derived vari-

ants were established after five consecutive ALA-PDT treatments [35]. However, in this case, the authors indicated that the obtained populations did not show resistant properties and that the response to new PDT-treatments was similar to that of the parental cells.

On the other hand, there are no reports employing cell sorting methodologies for studying resistance to PDT, but some studies have been performed employing cells that highly express defined resistant markers. This is the case of the ATP-dependent transporter ABCG2, which is expressed at different levels in many cell lines used in *in vitro* and *in vivo* tumor models for PDT, which may affect their phototoxic efficacy [49, 50]. In addition, recently, Yu and Yu [73] treated primary cultures from a head and neck cancer (HNC) tumor with ALA-PDT and they studied the photosensitizing effect on CSCs markers, particularly ALDH1. They observed that ALA-PDT treatment significantly down-regulated the ALDH1 activity and reduced the CD44 positivity and stem cell signatures expression (Oct4 and Nanog) in sphere-forming cells. The authors concluded that ALA-PDT effectively reduced CSC-like properties, including ALDH1 activity, CD44 positivity, self-renewal and invasion. These findings can be considered the first study in which different CSC markers have been evaluated and related with the response to PDT.

Finally, it should be noted that the level of PDT resistance observed is, in general, less than that reported for most drug-resistant cell lines. Although the knowledge of drug resistance mechanism is far from being understood, drugs are quite specific and there are usually a single or a few subcellular targets (DNA, enzyme, receptors), as a direct effect of the treatment on the amplification of a membrane-bound glycoprotein transport system, decreased repair of a specific target or altered pathways. However, there are numerous sites and types of injury associated with PDT, and overlapping mechanisms are therefore involved in PDT-cytotoxicity and resistance. Therefore, modifying the sensitivity of cellular PDT targets or repair systems would not be expected to produce the same degree of resistance as observed with chemotherapeutic drugs, which are associated with a limited number of targets or mechanisms of action. Hence, 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, different reports indicated that the relative resistance to PDT for the tumor cell lines is photosensitizer-specific.

Initial Characterization of PDT-Resistant Cells

The determination of structural, biochemical, molecular and/or functional differences between the parental and the PDT-tumor resistant cells is a main goal in order to provide mechanisms underlying altered susceptibility to PDT. This chapter included in this volume, as well as the review already published by Casas et al. [28] point out the different cellular and molecular characteristics of the PDT-resistant cells. Overexpression/mutations of growth factors and growth factor receptors, as well as of signal transduction proteins, lead to sustained proliferative and survival

signaling and to an aberrant proliferation, which contribute to PDT resistance. Some examples that could be tested as molecular markers are (i) “gain-of-function” gene alterations, such as the PI3K/Akt/mTOR and MAPK/ERK pathways [74–77] (ii) inactivating mutations in tumor suppressor genes, such as the *retinoblastoma (RB)*, *PTEN (phosphatase and tensin homologue deleted from chromosome 10)* and *P53* [78–81] (iii) alterations in the machinery of apoptosis or autophagy, including overexpression of anti-apoptotic proteins like Bcl-2, IAPs survivin and inactivation of pro-apoptotic genes such as genes encoding caspases or proapoptotic Bcl-2 members [82–86] (iv) oxidative and stress genes and proteins, such as hemeoxygenase (HO), heat shock proteins (HSP), superoxide dismutase (SOD), glutathione peroxidase [87–90] and (v) proteins related with ATP-binding cassette transporter [49–53]. Therefore, here we only emphasized some aspects to help in an initial characterization of these resistant cells compared to the parental population from which they have been isolated.

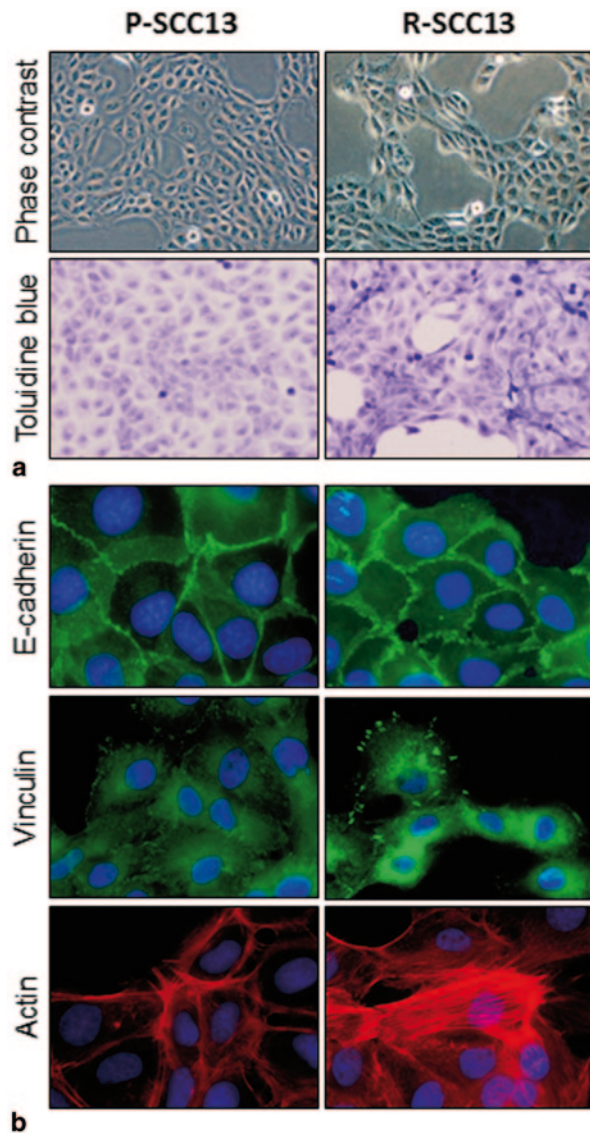
Cell Morphology and Population Characteristics

In general, the results obtained in different laboratories related with the changes in the morphology of isolated resistant cells, compared with the appearance of the parental cells, are homogeneous. However, the results reported on cell dynamic characteristics are contradictory. It has been described that PDT-resistant cells change their morphology in relation to that shown by the parental cells. The resistant cells isolated after treatment with Photofrin II from RIF-1 showed an increase in cell size compared to that of the parental cells [26, 34]. Nuclear size was also increased. Total cellular protein content was, as well, significantly higher than that of the parental cells. Plating efficiency of the 1 h PDT-resistant variants was similar to that of the parental RIF-1 cells, whereas in the case of 16 h the PDT-resistant variants plating efficiency was reduced to 36–43%. However, the cell doubling time for resistant and parental cell types was similar.

Similar results have been described for the resistant-PDT Clon 4 and Clon 8 (both isolated from LM3) in terms of protein content, being higher in the resistant clones (2-fold increase) [28]. However, the plating efficiency was significantly impaired (25–30%) in both Clon 4 and Clon 8 compared to LM3, as well as the growth rate, which was also significantly decreased in the resistant clones compared with the parental LM3 (3.5-fold lower in Clon 8 than the control). An increase in the latency time has been reported for Clon 8 cells compared to the parental LM3 cells [91].

Similarly, there were no substantial differences on cell size, plating efficiency and distribution of the cells in the cell cycle between the SCC-13 cells and the PDT-resistant variants (unpublished results from our laboratory). It has also been described that SCC-13 cells present a diverse morphology [36]. SCC-13 parental cells showed a polyhedral to fibroblastic with long prolongations morphology, which was also observed in the resistant isolated generations; however, these cells had a higher proportion of fibroblastic forms and the cell colonies formed were more

Fig. 5.2 Cell morphology of PDT-resistant cells compared to parental SCC-13 cells. **a** Resistant PDT-cells (R-SCC-13) show a more pronounced fibroblastic morphology compared to parental cells (P-SCC-13) when they are observed under phase contrast as well as after Toluidine blue staining. **b** E-cadherin expression is similar in both cell types, whereas higher expression of vinculin as well as higher amounts of thick stress fibers can be seen in the resistant SCC-13 population



expansive with respect to the parental populations (Fig. 5.2). This was also noted previously in Clon 4 and Clon 8 resistant cells. They exhibited a more fibroblastic, dendritic pattern, and a higher cell spreading than the LM3 parental line. At the subcellular level, electron microscopy showed that there were no noticeable differences on lysosomes and membranes among the lines, although the mitochondrial number per cell and per area was higher in both the resistant clones [28]. These results are in concordance with those previously published with RIF-1 cells [27, 92]. Thereby, the mitochondria in the resistant RIF-8A cells were smaller, while their

number per cell was higher than in the parental RIF-1 cells. In addition, the RIF-8A cells produced more ATP and demonstrated higher succinate dehydrogenase activity than the RIF-1 cells. The authors indicated differences in the efficacy and/or the mode(s) of energy production in the RIF-1 and RIF-8A and pointed out that, since the mitochondria are sensitive targets for porphyrin-mediated PDT [12, 93], the observed changes in structure and/or function (or both) of the mitochondrion may be involved in the PDT resistance seen in RIF-8A cells. In contrast, alterations related with the integrity and functionality of the mitochondria have been described in the established PDT-derived variants CL1-5/6A5, A375/3A5, and MDA-MB-231/1A5 isolated from CL1-5, A375, and MDA-MB-231, respectively. In this case, the mitochondrial membrane potential was significantly reduced. The authors indicated that, in these cells, the consecutive ALA-PDT treatments caused permanent mitochondrial damage in the established PDT-derived variants. In any case, these isolated variants are not considered as PDT-resistant cells [35].

In the human colon adenocarcinoma HT29 cells, PDT-resistant variants, selected from sequential PDT treatments by using different photosensitizers, showed downregulation in the mitochondrial genes coding for the 16S ribosomal RNA (rRNA) and nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4. The authors also found, in the PDT-resistant variants, an increased expression of the gene encoding the Bcl-2 protein and downregulation of the gene encoding the Bax protein, suggesting that both the altered expression in the mitochondrion and apoptosis-regulating genes contribute to PDT resistance [94]. Similarly, it has been recently reported in three human head and neck squamous carcinoma cell lines, (UMSCC1, UMSCC14A, and UMSCC22A), treated with silicon phthalocyanine (Pc4) as a mitochondria-targeted photosensitizer, that they responded differently [95]. UMSCC1 and UMSCC14A cells were more resistant than UMSCC22A cells to Pc 4-PDT-induced cell death. The authors indicated that this differential response was due to the expression of the mitochondrial protein mitoferrin-2 (Mfn2), an iron transporter of the mitochondrial inner membrane. PDT-sensitive cells expressed higher Mfn2 mRNA and protein levels compared with the PDT-resistant cells.

Nuclear Analysis

A higher degree of nuclear heterogeneity is generally present in cultured PDT-resistant cells. Also, long nuclear connections can be found between nuclei of cells in division. Giant nuclei (polyploidy) are also observed in higher proportion in resistant cells related to parental cells [26, 36, 92]. In addition, it has been described an increase in the number of cells with micronuclei in the resistant SCC-13 cells ($12\% \pm 2.8$) as compared to the parental SCC-13 cells ($3 \pm 1.3\%$) [36]. All these results would be in agreement with the high rate of abnormal divisions observed, particularly at anaphase or telophase, with chromosomal material present in the middle of the two cells [36]. Micronuclei presence has also been described in many different resistant cells, such as the hepatocellular carcinoma HepG2 cell line (subjected to etoposide treatment) [96] and the human endometrial adenocarcinoma HEC-1 cells (subjected to paclitaxel treatment) [97].

Analysis of the karyotype revealed that most of the parental RIF-1 cells were diploid or tetraploid (40 and 80 chromosomes) and contained some abnormal chromosomes. The resistant RIF-8A variant cell karyotype was inconsistent, being the most frequently observed presence of the polyploidies of 120 chromosomes [27]. In addition, using a comparative genomic hybridization array (aCGH), Gilaberte et al. [8] reported that both resistant and parental SCC-13 cells present 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 not present in the parental cells. These changes detected by aCGH on CCND1, EFGR and MAP3K1 were confirmed by western blot, suggesting that genomic imbalances related to CCND1, EFGR and particularly MAP3K1 could be involved in the development of resistance of SCC to PDT. Previous studies indicated that PDT can produce single and double strand breaks, sister chromatids exchanges, chromosome aberrations and mutagenic alterations [98–101], supporting the results described in the resistant populations and indicating 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 [102–106].

PS Accumulation and Subcellular Localization

It has been proposed that the differential response to PDT could be due to different PS accumulation in parental and resistant cells. Hence, Luna and Gomer [26] have found that, generally, the amount of PII per cell was slightly increased in the resistant variant CL-8 cells as compared to the parental RIF-1 cells, although CL-1 cells retained approximately one-half of the amounts of PII. In addition, CL-8 cells have a 1.5-fold increase PDT resistance and CL-1 cells exhibited even higher (4.5-fold) in survival cells following PII incubation. Therefore, the authors indicated that PDT resistance exhibited by the CL-1 and CL-8 cells was not due solely to decreases in PII accumulation.

Likewise, since the amount of ALA/MAL-converted PpIX might affect phototoxicity, the examination of whether the differential cytotoxicity was due to the different PpIX contents in resistant vs nonresistant cells has also to be taken into account. Therefore, ALA-PDT did not cause significant differences in phototoxicity between the parental cells and the PDT-derived variants from CL1–5, A375, and MDA-MB-231 cancer cells [35]. PpIX accumulation was very low in CL1–5 cells and did not change significantly as the ALA concentration was increased; meanwhile, MDA-MB-231 cells produced relatively high PpIX content. It appears that the differential ALA-converted PpIX content would explain the differential phototoxicity among the parental and the derived CL-5 and MDA-MB-231 variants.

In the case of LM3 cells, Casas et al. [28] found that the amount of porphyrins synthesized by LM3 cells normalized by cell number was not significantly different from the resistant sublines (Clon 4 and Clon 8), but when expressed on a per μg protein basis, the porphyrin synthesis was increased 2-fold in the parental line.

In addition, Milla et al. [36] did not find differences in the production of PpIX after incubation with MAL between the parental SCC-13 and the resistant isolated populations.

With respect to the subcellular localization of the PSs, Casas et al. [28] described the distribution of endogenously synthesized PpIX after incubation with ALA, in the LM-3 parental cells as well as in the resistant clones. The parental and resistant populations exhibited a similar cytoplasmic PpIX localization, including mitochondria, lysosomes, the cell membrane and the Golgi apparatus. Similarly, localization of Photofrin and ALA-induced PpIX in the parental RIF-1 tumor cells and in the RIF-8 resistant to Photofrin was similar [107]. In both cell types, PSs are located mainly in the mitochondria. They also evaluated the uptake kinetics of Photofrin alone and after coinubation with mitochondria-specific probes (10N-Nonyl acridine orange, NAO or rhodamine-123, Rh-123) showing a stronger colocalization of Photofrin, NAO and Rh-123 in RIF-1 than in RIF-8 cells. The authors indicated that the differences in this binding may account for the PDT resistance in RIF-8A cells. However, it should be emphasized that in both cell types the subcellular localization was mitochondrial. In addition, Mayhew et al. [33] described that the two resistant strains also isolated from RIF-1 cells treated with PPC or PHP did not show differences in the localization of the PSs comparing with the parental cells and neither in drug uptake. The authors concluded that in both PDT-resistant strains, the increased resistance could not be attributed to the intracellular sensitizer localization.

In agreement with the results described above, no major differences have been found in PpIX localization among the parental and the resistant SCC-13 cells. PpIX was localized in the plasmatic membrane in all analyzed populations, but very low fluorescence intensity was also detected into lysosomes and mitochondria and in cytoplasm. PpIX was also observed in vacuoles at longer incubation periods using also organelle markers, such as Mitotracker or Lisotracker. There is a problem when the concentrations and the incubation times are low since PpIX fluorescence is very difficult to detect by optical resources due the immediate photobleaching of the PS under the microscopy exciting light of 460–490 nm [36].

Therefore, taking altogether the published data, it is not clear that differences between parental and resistant cells in the subcellular localization of the PS even in its intracellular accumulation would be the cause of the differential response to PDT after identical treatment conditions. Nevertheless, several reports indicated 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 [51–53, 108, 109]. 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.

Cell Adhesion and Migration Abilities

Cell adhesion proteins play a crucial role in migration and invasion abilities of cancer cells. Therefore, the expression and distribution of the proteins implicated in

these processes would be also important to determine the resistance to PDT abilities of cancer cells [46, 47, 110–112]. Casas et al., [91, 113] showed that in mammary adenocarcinoma cells (LM3), E-cadherin is located at the plasma membrane connecting neighbor cells, but it is disorganized in Clone 4 and Clone 8 LM3-resistant cells. E-cadherin distribution was completely aberrant in the resistant clones, being situated in the numerous interdigitations which are present along cell to cell contacts. Similarly, β -catenin showed the same distribution pattern for E-cadherin in LM3 cells, being also disorganized in the interdigitations and showing a diffuse cytoplasmic distribution. In addition, the authors did not find significant differences in the expression of cell-substrate adhesion proteins β 1-integrin, vinculin, FAK and phospho-FAK in the resistant clones, compared to LM3 cells. However, the vinculin distribution was different; whereas in LM3-parental cell, vinculin was confined to the focal adhesion points, a diffuse cytoplasmic pattern was observed in the resistant clones. FAK distribution was both cytoplasmic and nuclear, whereas phospho-FAK was confined to the focal adhesion points, and no differences were found among the distribution in the cell lines. Related with cell adhesion and migration, actin microfilaments constitute a basic cytoskeletal element [114–116]. Thus, whereas long stress fibers situated at the basal plane were present in LM3, this organization became perturbed in Clone 4 and Clone 8 cells. In Clone 4 cells stress fibers were shorter and only a few of them were found in Clone 8 cells. A fine, quite regular and continuous cortical F-actin layer was present in LM3 cells, whereas it was more irregular in Clone 4 and a waved pattern of cortical actin was observed in Clone 8 cells. No significant differences in the adhesion of the three cell lines to the ECM proteins fibronectin and laminin were found, whereas Clon 4 and Clon 8 adhesion ability to Collagen I were 1.3 and 2-fold as compared to LM3, respectively.

Milla et al. [36] did not find strong differences in the expression patterns and levels of E-cadherin and β -catenin between resistant and parental SCC-13 cells (Fig. 5.2). They also evaluated the expression levels of cell-substrate adhesion proteins β 1-integrin, vinculin, FAK and phospho-FAK. In resistant cells vinculin and phospho-FAK showed a distribution in the center and in the cellular periphery, while in parental cells they were mainly in the center. Vinculin was localized at the end of the stress fiber in the three studied populations (Fig. 5.2). By western blot analysis, they observed that resistant cells had higher expression of β 1-integrin, vinculin and phospho-FAK with respect to the parental cells. The pattern of the actin stress fibers showed that, in the resistant SCC-13 cells, F-actin was highly expressed in cortical regions and many cells showed conspicuous stress fibers as compared to parental cells. Unpublished results obtained in our laboratory revealed higher adhesion ability to Collagen I of the SCC-13 resistant cells compared to that of the parental cells (1.5-fold).

Motility is a key factor in the regulation of cancer cell invasion [42, 116, 117]. Therefore, to test this property, characterizing PDT-resistant cells is also important. There are several studies with different cell lines and PSs indicating that motility and invasion abilities are reduced after PDT. These studies include, for instance, the head and neck cancer cell lines KJ-1 and Ca9-22, treated with ALA [118], glioma spheroids obtained from human U373 and A172 cell lines treated with ALA [119],

nasopharyngeal carcinoma KJ-1 cell line treated with tetrahydroxyphenyl chlorine [120] or human ovarian cancer HO-8910 treated with hypocrellin B [121]. In relation with the PDT-resistant cells, Tsai et al. [35] indicated that the migration ability was permanently affected in the established PDT-derived variants CL1–5, A375 and MDA-MB-231 cancer cells. In fact, by using the scratch wound assay, the authors found a significant reduction in migration in those survived from ALA-PDT, suggesting that the photodamage induced by ALA-PDT caused the suppression of cell migration ability in these cells. In addition, invasion is also affected in ALA-PDT-derived CL1–5, A375 and MDA-MB-231 variants [35]. By using the Matrigel assay, Casas et al. [91] did not find significant differences between the LM3 and resistant clones (4 and 8). The authors also tested the chemotaxis or directional migration using control inserts, and saw that 100% of LM3 cells migrated through the porous membrane, whereas only the $38 \pm 8\%$ and $73 \pm 0\%$ of Clones 4 and 8, respectively, were able to migrate, concluding that the resistant clones presented lower invasion abilities than the parental LM3 cells. The authors related the decreased abilities of the resistant cells with the alterations in the expression of adhesion proteins and microfilaments indicated above.

On the contrary, the ability of migration and closing wounds evaluated by the scratch wound assay in parental SCC-13 and in the PDT-derived resistant variants indicated that, whereas at early time (4 and 8 h) after starting the assay there were no differences in the migration capacity, however, the resistant cells showed higher capacity of closing wounds at longer times (12 and 24 h) compared to the parental cells [36].

All these differences in the results obtained the migration and invasion abilities of the resistant variants, these could be due to different factors, including (i) the cell line, (ii) the PS or prodrug used and (iii) the way of selecting resistant cells.

Tumor Induction and Metastatic Abilities in Mice

There are many animal models to study drug resistance with advantages and disadvantages as reviewed by Rottenberg and Jonkers [122] and Politi and Pao [123]. In general, cancer cell lines are injected into immunodeficient mice for testing tumorigenicity and metastatic abilities of cultured cancer cells. This is the strategy used by the investigators to test the characteristics of resistant cells to PDT. Luna and Gomer [26] evaluated the tumorigenic ability of the two PDT-resistant variants of the RIF-1 mouse tumor cell line obtained by repeated treatment with PII after two incubation times (16 h and 1 h). The authors injected variable number of cells (10 up to 10^6) into the flanks of immunocompetent C3H mice and found that the number of cells required to produce palpable tumors in 50% of inoculated mice (latency time) was 10–20 for parental RIF-1 cells but it was higher (between 5×10^4 and 5×10^5) for the PDT-resistant variants. Similar results were obtained when athymic “nude” mice were used as host animals. Tumor-doubling time in C3H mice was similar for the parental RIF-1 line and for the 16-h P-II PDT-resistant variants (2–2.8 days).

However, the 1-h PDT resistant strains had increased doubling times, ranging from 3.9–4.6 days.

Similar results related to the ability to form tumors by resistant cells were found by Casas et al. [91]. They evaluated the ability of the parental LM3 cells and the PDT-resistant clones (Clon 4 and Clon 8) to grow subcutaneously in mice to form primary tumors and spontaneously to metastasize to the lung. An amount of 10^5 or 10^6 cells were injected in immunodeficient BALB/c mice. The authors found that tumor take (percentage of mice that developed palpable tumors at latency time) was decreased in the resistant clones compared with the LM3 line, most markedly in Clon 8. When 10^5 cells were injected, 30% of mice developed tumors, whereas no tumors were developed by the resistant clones. Increasing the amount injected to 5×10^5 cells, tumor take was 100% for LM3 cells, 60% for Clon 4 and 30% for Clon 8. Further increasing the amount injected to 10^6 cells, 100% of mice injected with LM3 and Clon 4 developed tumors, and only 60% of mice injected with Clon 8 did. The growth rate was also significantly decreased in Clon 4 compared with LM3, and Clon 8 growth delay was even more marked, 3.5-fold lower than the control. Latency time was similar for LM3 and Clon 4 whereas it was markedly longer for Clon 8 cells ($p < 0.001$). They also evaluated the spontaneous lung metastasis induced by LM3 and resistant clones and whereas LM3 cells metastasized to the lung in a tumor-size dependant way, Clones 8 and 4 almost did not induce nearly any metastasis at all. Only one small lung metastasis was found in one Clon 4 in the 7–19 mm tumor diameter range, whereas Clon 8 cells did not induce any metastasis at all. The authors related these results with 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.

However, in the case of the article recently published by our laboratory [8], the results obtained after subcutaneous inoculation in immunodeficient mice of the squamous cell carcinoma SCC-13 cells and the obtained PDT-resistant populations were different (Fig. 5.3). Both the parental and the resistant cells formed progressively growing tumors, but the tumors induced by the PDT-resistant cells were bigger than those induced by the parental cells. Also the number of tumors was significantly higher in mice injected with resistant cells compared to those induced by parental cells. The differences between the mean number of tumors developed per mouse injected with the parental and resistant cells were statistically significant on days 15 and 30. We also evaluated the histological characteristics and whereas the tumors induced by parental SCC-13 cells were mostly well or moderately differentiated squamous cell carcinomas, those induced by PDT-resistant cells were mostly moderately or poorly-differentiated SCC, formed by atypical keratinocytes with nuclear pleomorphism even infiltrating skeletal muscle fibers (Fig. 5.3). The metastatic abilities of the resistant SCC-13 cells were not evaluated. Although not performed with PDT-resistant cells, previous results published by Momma et al., [124] 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, found that PDT produced a significant increase in the mean number

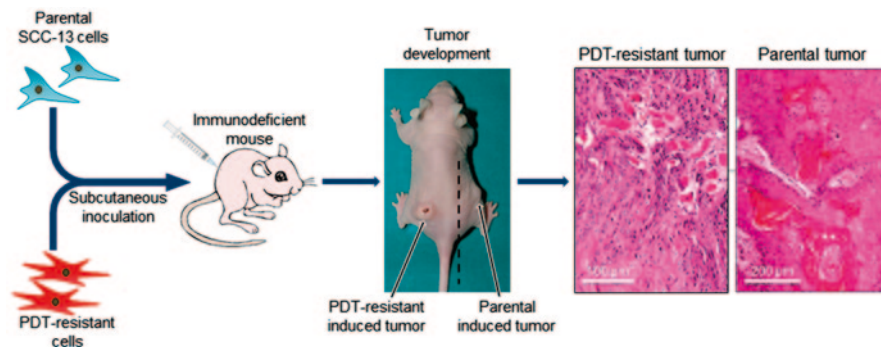


Fig. 5.3 *In vivo* tumor development after inoculation of parental squamous carcinoma cells SCC-13 cells and PDT-resistant cells in immunosuppressed mice. Parental and PDT-resistant SCC-13 cells are injected in the right and in the left flanks of the mice, respectively. The tumor induced by the resistant variant is bigger compared to that of parental. Then, histopathological analysis revealed that the tumor induced by the resistant variant presents characteristics of poorly differentiated squamous cell carcinoma with cellular atypia and aberrant mitotic cells. In addition, squamous cells infiltrating the skeletal muscle can be observed. The tumor induced by the parental cells showed characteristics of well differentiated squamous cell carcinoma with dyskeratotic cells and keratin accumulations

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 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 [26] and by Casas et al., [28] 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 cloning methodology, the optimal resistant clones to study tumorigenicity in mice were not selected, whereas in the resistant cell population cells with different tumorigenic abilities are present. Obviously other factors can also contribute to the differences obtained, included the cell line, the PS and the experimental conditions.

Conclusions

Resistance constitutes a relevant unsolved problem in cancer therapy. Cancer cell culture assays, as well as mice models, are excellent tools available to diagnose intrinsic and acquired resistance, which may develop rapidly as the results of repeated treatments. Thus, a current challenge in PDT is modelling, both in cellular and animal systems, the characteristics associated to tumor resistance, that may reveal useful information from the molecular basis of intrinsic and acquired resistance to PDT.

No Conflict Statement “No potential conflicts of interest were disclosed.”

Acknowledgments The work was supported by Ministerio de Economía y Competitividad (MINECO, FIS PI12/01253), and Comunidad de Madrid (CAM, S2010/BMD-2359), Spain. AZ is recipient of a PhD contract by CAM and SL has a fellowship by UAM (Universidad Autónoma de Madrid).

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