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Ras-transfected human mammary tumour cells are resistant to photodynamic therapy by mechanisms related to cell adhesion

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ABSTRACT

Aims: Photodynamic therapy (PDT) is a treatment modality for several cancers involving the administration of a tumour-localising photosensitiser (PS) and its subsequent activation by light, resulting in tumour damage. Ras oncogenes have been strongly associated with chemo- and radio-resistance. Based on the described roles of adhesion and cell morphology on drug resistance, we studied if the differences in shape, cell-extracellular matrix and cell-cell adhesion induced by Ras transfection, play a role in the resistance to PDT.

Materials and methods: We employed the human normal breast HB4a cells transfected with H-RAS and a panel of five PSs.

Key findings: We found that resistance to PDT of the HB4a-Ras cells employing all the PSs, increased between 1.3 and 2.5-fold as compared to the parental cells. There was no correlation between resistance and intracellular PS levels or PS intracellular localisation.

Even when Ras-transfected cells present lower adherence to the ECM proteins, this does not make them more sensitive to PDT or chemotherapy. On the contrary, a marked gain of resistance to PDT was observed in floating cells as compared to adhesive cells, accounting for the higher ability conferred by Ras to survive in conditions of decreased cell-extracellular matrix interactions.

HB4a-Ras cells displayed disorganisation of actin fibres, mislocalised E-cadherin and vinculin and lower expression of E-cadherin and β1-integrin as compared to HB4a cells.

Significance: Knowledge of the mechanisms of resistance to photodamage in Ras-overexpressing cells may lead to the optimization of the combination of PDT with other treatments.

1. Introduction

Photodynamic therapy (PDT) is a treatment modality available for the palliation or eradication of several cancers. PDT involves systemic or topical administration of a tumour-localising photosensitiser (PS), and its subsequent activation by visible light to result in reactive oxygen, mainly singlet oxygen, inducing photodamage to the tumour [1,2].

Numerous organelles have been described as targets for the cytotoxic effects of singlet oxygen including mitochondria, lysosomes, Golgi apparatus, plasma membrane and nuclei and therefore, PSs display different localisation sites. Cytoskeletal structures are also affected by photosensitisation [3].

Abbreviations: ALA, 5-aminolevulinic acid; ARPCD, adhesion-regulated programmed cell death; AO, acridine orange; CAM-DR, cell adhesion-mediated resistance; Ce6, Chlorin e6; ECM, extracellular matrix; MC540, Merocyanine 540; m-THPC, meta-tetrahydroxyphenylchlorin or Temoporfin; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide); PS, photosensitiser; PDT, photodynamic therapy; PII, Photofrin II or Porfimer sodium.

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Currently, there are a large number of PSs at various stages of clinical trials. Photofrin II or Porfimer sodium (PII) was approved by the FDA for advanced obstructive oesophagal cancer and the treatment of early-stage non-small-cell lung cancer. M-tetrahydroxyphenylchlorin (m-THPC) was approved in Europe for advanced head and neck squamous cell carcinoma, and Verteporfin was approved by the FDA for the treatment of wet age-related macular degeneration [4].

In the last years, the design of new PSs has been focused to maximize the absorption of light at wavelengths of higher tissue penetration. Chlorin e6 (Ce6) is structurally similar to porphyrins with its enhanced absorbance of red light [5]. M-THPC which is also a chlorin differs from its related porphyrin only in the presence of a centre of saturation which improves its photoactivity [6]. Verteporfin exhibits an absorption maximum at 690 nm.

Among the non-porphyrin PSs, some dyes have been used in PDT with relative efficacy. Merocyanine 540 (MC540) is a negatively charged heterocyclic chromophore which has been used in PDT of leukaemias and solid tumours [7]. It has been suggested that MC540 enter the cell but locate at the membranes, therefore the membrane lipids are considered to be photodamage targets [8]. Acridine orange (AO) is a fluorescent dye that interacts with nucleic acids, accumulates in lysosomes and after illumination induces nucleic acid photodamage [8,9].

The RAS genes (HRAS, KRAS, and NRAS) are the most frequently mutated oncogene family in human cancer [10]. Ras is notoriously difficult to target, due to its structure and intrinsic activity, therefore, drug and radiation Ras-resistance remains a considerable pharmacological problem [11,12].

Expression of mutant RAS oncogenes is known to increase the motility, invasiveness and metastatic potential of cells [13]. Oncogenic Ras activation regulates integrin expression, affinity and avidity of integrins for ECM [14], E-cadherin expression [15,16], and cytoskeleton modulation [17] among other multiple signals.

In previous work, we have shown that the human normal breast HB4a cells transfected with the oncogene H-Ras are resistant to PDT with the pro-PS 5-aminolevulinic acid (ALA) [18]. In addition, whereas HB4a cells spread more on the substratum and display a typical epithelial appearance, Ras-transfected ones display round morphology, with fewer adhesion contacts and exhibit certain differences in cytoskeleton proteins that are modified after ALA-PDT [33]. The relationship between resistance to PDT and cell adhesion and cytoskeleton has been previously studied and reviewed by us [19].

Adhesion to the extracellular matrix (ECM) is necessary for the survival of epithelial cells. Disruption of this mechanism triggers the process of anoikis, a special type of apoptosis. However, in processes such as migration or metastasis in which cells are not connected to the ECM, cells are protected from anoikis. Integrins are the main proteins responsible for transmitting signals from the ECM, but even though integrin-mediated cell-ECM anchorage has been long recognized as crucial for epithelial cell survival, the *in vivo* significance of this interaction is not clear [18].

The morphology of a cell is determined *in vivo* by the ECM and *in vitro* by the substratum, and it is an important factor in the sensitivity to chemical injuries [19]. The cell type, the presence of other cells, cell shape, tissue topology and the type of adhesive interaction with ECM influence the balance between the life and death of the cell. In normal intestinal cells, malignant transformation induced by the insertion of RAS provokes resistance to anoikis or apoptosis induced after cell detachment [20]. Oncogenic activation of the EGFR/Ras pathway in integrin mutant cells also rescues them from apoptosis while promoting their extrusion from the epithelium [18], a process which contributes partly to the resistance to chemotherapy conferred by Ras.

This work aimed to test the impact of Ras activation on the outcome of PDT of a mammary cell line employing various photosensitisers with different intracellular localisation and its relationship with cell adhesion, integrins and cytoskeleton proteins.

2. Materials and methods

2.1. Chemicals

PII and Verteporfin (Visudyne®) were purchased from QLT, Inc. (Vancouver, Canada), MC540, Basic Orange 14, 3,6-Bis(dimethylamino) acridine hydrochloride zinc chloride double salt (AO) and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) (MTT) was obtained from Sigma-Aldrich (St. Louis, USA). Meta-tetrahydroxyphenylchlorin (m-THPC) (Temoporfin, Foscan®) was purchased from Biolitec Pharma Ltd., Ireland. Ce6 was obtained from Frontier Scientific Inc., Logan UT, USA. Cisplatin and Doxorubicin were obtained from Sigma-Aldrich (St Louis, USA). Methotrexate, 5-Fluorouracil, and Mitomycin C were donations from Kampel Martian (Buenos Aires, Argentina).

2.2. Cell lines and cell culture

The HB4a cell line is a clonal, non-transformed, non-tumourigenic line derived from reduction mammoplasty tissue. It was one of a panel of immortal cell lines developed by the transduction of the SV40-derived recombinant viral oncogene mutant tsA58-U19 [20]. HBAa r4.2 (HB4a-Ras) cells were generated by transfecting HB4a parental cells with the plasmid pEJ containing a 6.6-Kb genomic H-Ras (VAL/12 Ras) sequence [21].

The cells were maintained at 37 °C in an atmosphere containing 5 % CO_2 in RPMI 1640 medium with L-glutamine 40 μg/ml, 10 % fetal bovine serum, 5 μg/ml hydrocortisone and 5 μg/ml insulin. Ras expression was checked periodically.

2.3. Chemical extraction of photosensitisers

Cells were exposed to different PSs concentrations in media without serum. In previous experiments, different incubation times were employed, and PS concentration ranges have been chosen based on the MTT cytotoxicity assay. PSs were extracted by different solvents (taken from literature) after 3 washes with PBS and afterwards, fluorescence was measured in a Perkin Elmer LS 55 fluorimeter employing the maxima excitation and emission light wavelengths (Table I).

2.4. PDT treatment

Cells were exposed to the different PSs in medium without serum employing incubation times and concentrations were chosen according to the extraction experiments described above. The criteria for choosing PS concentrations to perform PDT experiments was a drug dose that induced intracellular fluorescence >3 times over basal autofluorescence values, and that the light doses required to kill 50 % of the cells were not higher than 100 mJ/cm^2. In the cases where the PS accumulation was not equal for both cell lines, the maxima concentrations leading to equal intracellular PSs values were chosen. After PS exposure, the cells were irradiated for different periods from below. Afterwards, the medium was replaced by medium containing serum and the plates were incubated for 19 h at 37 °C to let the photodamage occur, and the MTT assay was performed. The light source employed was a bank of two fluorescent lamps (Osram L 36W/10) [27] and the power density was 0.5 mW/cm^2. Results were expressed as the percentage of the non-illuminated control. Lethal doses 50 (LD_{50}s) were defined as the light doses (J/cm^2) necessary to kill 50 % of cells.

2.5. MTT assay

Cell viability was documented by the MTT assay [28]. Absorbance was quantified in a Spectracount plate reader (Packard, USA).

Table I
Conditions for PSs extraction.

	PII	Verteporfin	MC540	AO	m-THPC	Ce6
Incubation time (h)	2	3	2	2	2	2
Solvent	5 % HCl	MeOH:H_2O (1:1)	EtOH:acetic acid:H_2O (70:5:25)	MeOH:acetic acid: H_2O (70:5:25)	MeOH:DMS O: (4:1)	MeOH
Excitation wavelength [nm]	406	420	520	500	423	405
Emission wavelength [nm]	604	690	580	592	657	570
Reference	[22]	[23]	[24]	[24]	[25]	[26]

2.6. Immunostainings

Cells were grown on coverslips, fixed, and stained for immunodetection as described before [29]. Primary antibodies anti-E-cadherin, vinculin, and α-tubulin were used at 1:100 and FITC-labeled secondary antibodies at 1:500. For F-actin detection, cells were incubated with TRITC-phalloidin. All preparations were counterstained with Hoechst 33258 and mounted in Prolong-GOLD (Thermo Fisher).

Microscopic observation and photography were performed in an Olympus photomicroscope BX51, equipped and the corresponding filters. We have also employed a confocal laser scanning microscope Leica TCS SP2 using a 63× objective, and laser excitation at 488 nm.

2.7. PS intracellular localisation

Cells were grown on coverslips and exposed to the PSs under the optimal conditions for each one, and afterwards, they were washed with PBS and mounted on glass slides. Spontaneous fluorescence emission was observed in a fluorescence confocal microscope (Nikon D-Eclipse C1) employing 488 and 544 nm excitation and BP filters of 515/30 nm and 570 nm. Auto-fluorescence of control cells was also documented.

2.8. Immunoblots

Lysates were prepared from subconfluent monolayers and proteins were resolved by SDS-PAGE as described previously [30]. The conditions employed for primary antibodies were as follows: anti-E-cadherin (1:200), β1-integrin (1:200), vinculin (1:400), α-tubulin (1:1000) and G-actin (1:10000), (Santa Cruz Biotechnology, Inc.), 1 h at room temperature. A secondary anti-mouse antibody (Vector Laboratories, Burlingame, CA) was used at 1:10,000 and incubated for 1 h. Rainbow markers (10–250 KDa; Amersham International, USA) were used for molecular weight determination. Developed films were analyzed using ImageJ software [31] to quantify band density.

2.9. Adhesion assay to ECM proteins

96-Well plates were coated overnight at 4 °C with ECM proteins: fibronectin, vitronectin, laminin, collagen I and IV dissolved in pH 8.8 PBS in concentrations set in previous experiments. One hour before the beginning of the experiment, the ligands were discarded and unspecific sites were blocked by a 0.5 % BSA solution. Controls exposed to 40 μg/ml BSA were included. A cell suspension (5×10^5 ml^{-1}) in BSA-containing medium was seeded on the coated wells and incubated for 40 min. at 37 °C. Afterwards, 3 PBS washes were carried out to remove non-adherent cells, and the adherent ones were fixed with cold methanol and stained with 0.5 % crystal violet. Adhered cells were quantified by absorbance at 560 nm after solubilization in 2 % SDS.

2.10. PDT of cells in suspension

Cells were plated in Petri dishes and exposed to PII under the conditions established before. Afterwards, they were trypsinized, centrifuged and placed in 6-well plates, illuminated and shacked every 3 min to avoid cell attachment. Afterwards, the cells were left to adhere overnight and an MTT assay was performed.

2.11. Antineoplastic drugs IC_{50} determinations

Cells were used 48 h after plating and exposed for 6 h to Doxorubicin, Cisplatin, Mitomycin C, 5-Fluorouracil and Methotrexate. Immediately after treatment, cell viability was measured employing the MTT method. The half-inhibitory concentrations of cell growth (IC_{50}s) were calculated from the abscissa intercept from logistic curves constructed by plotting cell survival (%) *versus* drug concentration (μM).

2.12. Statistical treatment

The values in the figures and tables were expressed as mean ± standard deviations of the mean. For statistical analysis, Student's *t*-test or ANOVA followed by Tukey's tests were performed with GraphPad Prism 6 software. P values < 0.05 were considered significant.

3. Results

3.1. Ras-expressing cell lines show decreased sensitivity to common PSs used in PDT

Dose-response experiments were performed to establish the optimal dosage of PSs in darkness for HB4a and its counterpart Ras-transfected cells. Concentrations higher than 100 μM PII, 1 mM Verteporfin, 30 μM MC540, 110 μM AO, 7 mM m-THPC and 10 μM Ce6 turned out to show statistically significant toxicity (data not shown). In all subsequent PDT experiments, non-lethal concentrations of PSs were used.

To evaluate the sensitivity to PDT employing different PSs in HB4a (wt) and Ras-expressing cells, we incubated the cells with different concentrations of PII, MC540, AO, Verteporfin, m-THPC and Ce6 (Figs. 1A, C, 2A, C, 3A and C). The intracellular accumulation of all PSs increased for both cell lines as a function of concentration, and we chose concentrations of PSs that led to equal values of phototoxicity. However, when we evaluated cell toxicity, we found that, in general, HB4A-Ras cells were less sensitive than wt cells to phototoxicity generated by the photosensitisers tested.

Where Ras-transfected cells were significantly more resistant to PDT employing PII, MC540, Verteporfin, m-THPC, Ce6 and m-THPC, the response employing AO was not significantly different between HB4a and HB4a-Ras (Table II).

3.2. HB4a-Ras transfected cells exhibit also a certain degree of resistance to antineoplastic drugs

To explore the resistance of Ras-transfected cells to antineoplastic drugs, we exposed HB4a and HB4a-Ras cells to different concentrations of Mitomycin C, Doxorubicin, Methotrexate, Cisplatin and 5-Fluorouracil (Table III) and we found that the Ras-transfected cells were significantly more resistant to the 5 chemotherapeutic drugs assayed.

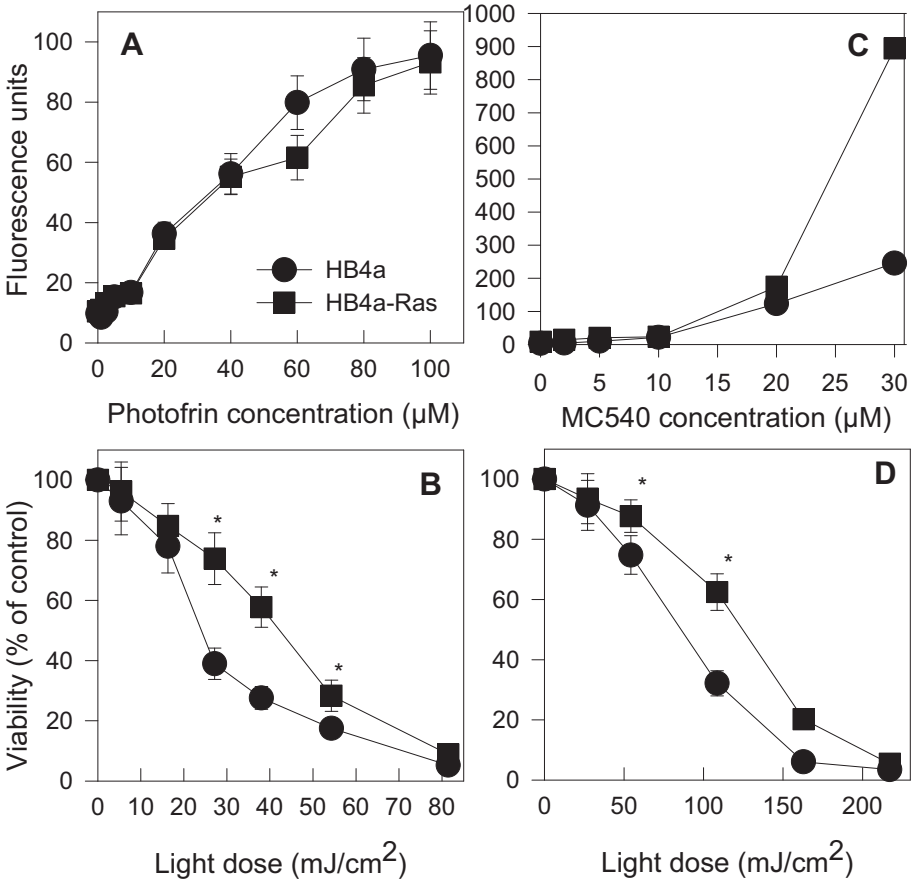

Fig. 1. PII and MC540 intracellular accumulation and response to PDT of HB4a and HB4a-Ras cells. Intracellular accumulation of PII (A) or MC540 (C) after 2 h exposure of cells to different PS concentrations. Survival after PDT mediated by PII or MC540 after 2 h exposure to 10 μM PII (B) or 20 μM MC540 (D) and different light doses was shown. Data represent the mean ± SD for three independent experiments performed in duplicates. *$p < 0.05$ between HB4a-and HB4a-Ras (ANOVA followed by Tukey's test).

3.3. Suspension culture renders cells more resistant to PDT, particularly the Ras-transfected ones

To find out a possible correlation between adhesion and PDT resistance, we illuminated in suspension HB4a and HB4a-Ras cells previously exposed to PII (Fig. 4). The resistance to PDT was significantly increased in both cell lines illuminated under suspension as compared to the cells illuminated under adhesion. LD_{50} of floating cells was increased 5-fold for HB4a wt (from 23 mJ/cm^2 (adhesion) to 110 mJ/cm^2 (suspension)) and 10-fold for HB4a-Ras (from 41 mJ/cm^2 (adhesion) to 400 mJ/cm^2 (suspension)).

Similarly, PDT employing MC540, AO, Verteporfin, m-THPC and Ce6 on suspended cells revealed that the LD_{50}s were increased 3.5 to 5-fold as compared to adherent HB4a cells and around 8 to 10-fold as compared to adherent HB4a-Ras. (Table IV).

3.4. PSs distribution in wt and Ras-transfected cells

Fig. S1 shows the subcellular distribution of the PSs studied. In general, HB4a cells spread more in the substrate while the Ras-transfected ones exhibit a rounded pattern with fewer adhesion sites to the substratum. All of the PSs except for MC540 show similar localisation in both cell lines.

MC540 appears to be localised in the Golgi apparatus, mitochondria and ER in Ras-transfected cells. On the other hand, mainly vesicles of red fluorescence are observed in HB4a cells and very few of these were visualized in HB4a-Ras cells.

3.5. Expression of E-cadherin, vinculin, F-actin, β1-integrin and α-tubulin in HB4a and HB4a-Ras cells

Quantification of Western blot bands reveals that vinculin expression was similar in both cell types whereas E-cadherin and β1-integrin expression was 1.4-fold and 2.1-fold upregulated respectively in HB4a-Ras cells (Fig. 5). In addition, actin and tubulin were employed as loading controls, and both cytoskeleton proteins displayed equal expression in the studied cell lines. β3 integrin was not detected in either of the cell lines. Original gels are displayed in the Supplementary material.

3.6. Localisation of E-cadherin, vinculin, β-actin and α-tubulin in HB4a and Ras-transfected cells

We have previously observed that HB4a cells spread more on the substrate and are more fibroblast-like as compared to the HB4a-Ras cells which are more rounded and epithelial. However, there are no differences in cell volumes among both cell types in suspension, showing that cytoskeleton and/or adhesion proteins may be involved in shape differences [32]. Therefore, in this paper, we studied the localisation of some cytoskeleton and adhesion proteins (Fig. 6).

In micrographs of HB4a cells, we can observe large actin stress fibres organised along the cytoplasm. Ras-transfected cells reveal the presence of a thick cortical actin rim and many cells show stress fibres mainly organised at the cell periphery, assembling in actin microspikes. On the other hand, we did not find any significant differences between both cell types after α-tubulin staining.

Whereas HB4a cells exhibit normal E-cadherin distribution on the cell-cell contacts, in Ras-transfected cells the distribution is disrupted with many interdigitations appearing along the cell-cell contacts. In

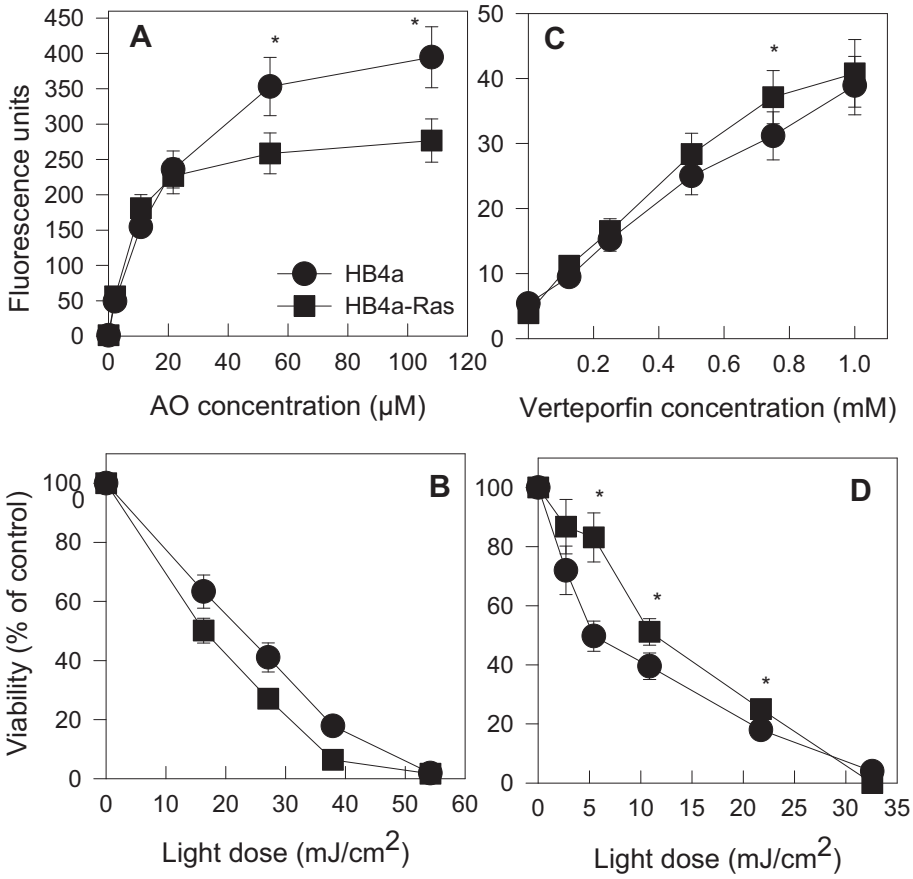

Fig. 2. AO and Verteporfin intracellular accumulation and response to PDT of HB4a and HB4a-Ras cells.

Intracellular accumulation of AO (A) or Verteporfin (C) after 3 or 2 h exposure respectively. Survival after PDT mediated by AO (2 h at 20 μM) (B) or Verteporfin (3 h at 0.5 mM) (D). Data represent the mean ± SD for three independent experiments performed in duplicates. *$p < 0.05$ between HB4a-and HB4a-Ras (ANOVA followed by Tukey's test).

addition, fewer cell-cell contacts are visible in HB4a-Ras cells.

On the other hand, large actin stress fibres organised throughout the cytoplasm are observed in HB4a-wt cells. Ras-transfected cells reveal the presence of a thick cortical actin rim and many cells show stress fibres mainly organised at the cell periphery, assembling in actin microspikes. Finally, we did not find any significant differences between both cell types after α-tubulin staining.

3.7. HB4a-Ras cells show lower adhesion to ECM proteins

Fig. 7 shows the profile of adhesion of HB4a and HB4a-Ras cells to different substrates. HB4a cells bind more strongly to the ECM proteins fibronectin, collagen I and IV and laminin. On the other hand, the binding was equal to vitronectin for both cell lines.

In addition, whereas HB4a cells adhere to all the ECM proteins in a ligand-concentration-dependent manner, HB4a-Ras displays this feature only with fibronectin and vitronectin.

4. Discussion

In the present study, we found that the H-Ras-transfected cells are more resistant to PDT with several PSs with different structures and localisation. The degree of resistance varied between 1.3 and 2.5, being Ce6, PII, Verteporfin and m-THPC the PSs inducing the higher resistance, whereas the lower resistance was induced by the non-tetrapyrrolic compounds AO and MC540. Even when PSs with different efficiency of photosensitisation were employed [33], we did not find any correlation between the photodamage degree and PDT resistance. In addition, Ras-mediated resistance seems to be extended to other non-free radicals-mediated therapies such as chemotherapy.

Photofrin, Verteporfin, m-THPC and Ce6 induced similar intracellular PS accumulation in HB4a and HB4a-Ras cells within all the concentration range analyzed. On the contrary, AO and MC540 induced equal intracellular accumulation at low concentrations, whereas, at high concentrations, HB4a-Ras cells accumulated higher amounts of MC540 and lower amounts of AO. However, we have carried out PDT in conditions of equal PS intracellular concentrations for both cell lines, thus showing that the mechanism of resistance is not related to differences in PS accumulation. We have previously found that the HB4a-Ras cell line was also more resistant to PDT employing endogenously produced Protoporphyrin IX as compared to HB4a. However, the mechanism was not related to PS accumulation [18].

The general mechanism of resistance to PDT of these Ras-transfected cells is very likely to be due to cell survival responses. It was reported that oncogenic activation of H-Ras in PAM212 murine keratinocytes can prevent photodynamic death induced by immunological disruption of E-cadherin adhesion and that H-Ras/PI3K/Akt signalling plays a key role in cell survival, concomitantly with resistance of Pam212 Ras-transfected cells to cell-substrate detachment after PDT [34].

However, resistance to PDT does not appear to be a general feature in Ras-transfected cells. Pazos et al. [35] did not find differences in the response to PDT of the EC endothelial cell line and its counterpart transfected with the EJ-RAS oncogene employing Aluminium-phthalocyanine. Moreover, bifunctional alkyl-modified porphyrins were able to target KRAS and NRAS G4 RNA structures upon illumination and to down-regulate the expression of these oncogenes [10].

The endoplasmic reticulum, Golgi apparatus and mitochondria are the preferential sites of tetrapyrrolic PSs [36]. On the other hand, AO accumulates in lysosomes and nucleic acids. MC540 has been reported to enter into the cells but accumulates at the membranes in the endoplasmic reticulum and Golgi apparatus [8]. In the present work, we found similar intracellular localisation in the HB4a and HB4a-Ras cells.

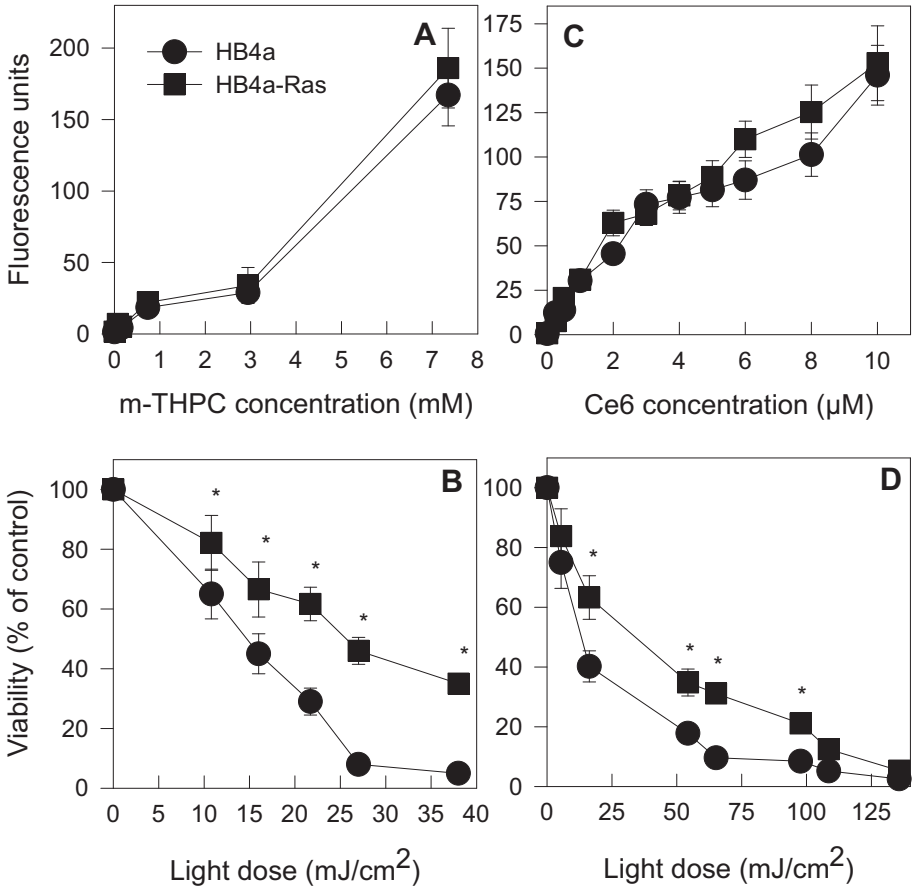

Fig. 3. m-THPC and Ce6 intracellular accumulation and response to PDT of HB4a and HB4a-Ras cells. Intracellular accumulation of m-THPC (A) and Ce6 (C) after 2 h exposure. Response to PDT was documented after 2 h exposure to 7 mM m-THPC (B) or 5 μM Ce6 (D) and different light doses. Data represent the mean ± SD for three independent experiments performed in duplicates. *$p < 0.05$ between HB4a- and HB4a-Ras (ANOVA followed by Tukey's test).

Table II
LD_{50}s (mJ/cm^2) of HB4a and HB4a-Ras exposed to PDT employing different PSs.

	PII	MC540	AO	Verteporfin	m-THPC	Ce6
HB4a	23	73	17	6.2	15	12.5
HB4a-Ras	41	120	22	12.2	27	32

Table III
CI_{50} (μM) of antineoplastics in HB4a and HB4a-Ras cells.

	HB4a	HB4a-Ras
Doxorubicin	2.3 ± 0.3	5.2 ± 0.4
Cisplatin	404 ± 32	473 ± 51
Mitomycin C	103 ± 14	142 ± 11
5-Fluorouracil	1212 ± 133	1654 ± 171
Methotrexate	63 ± 8	91 ± 8

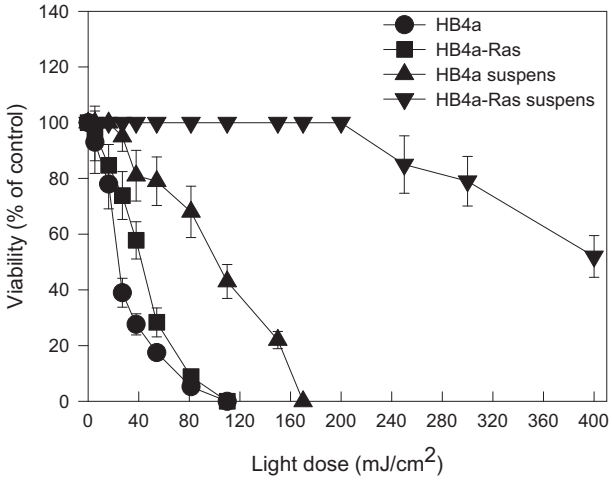

Fig. 4. PII-PDT in suspension-cultured cells.
Adherent cells were exposed for 2 h to 10 μM PII, and after trypsinization, illuminations were performed under suspension conditions. Data represent the mean ± SD for three independent experiments performed in duplicates. Afterwards, the percentage of viable cells was determined after 19 h of adherent culture.

However, cell membrane vesicles of MC540 were mainly found in HB4a but not in HB4a-Ras, probably related to the differences in cytoskeleton between both cell lines. It was previously described that MC540 was capable of rapidly releasing cytoskeleton-free vesicles from red blood cells [37].

Whereas HB4a cells spread more on the substratum, Ras-transfected ones are more rounded, with fewer focal adhesion contacts. However, an inspection of detached cells reveals that cell size is similar in both lines [18]. Due to the higher spreading observed in the parental cells, the appearance of the PSs is that of higher fluorescence signal in HB4a-Ras cells, however, this is not correlated either with the data of chemical extraction or the microscopic inspection of detached cells (data not depicted).

It has been reported that photodamage is tightly related to cell adhesion processes. However, there is no consensus on the kind of impact conferred by the therapy. Therefore, impairment, no change, or increased adhesion to plastic, ECM or endothelial cells and even disruption of ECM have been observed after PDT treatment [38–42,43]. PDT can also inhibit cell adhesion and affect integrin signalling without modifying cell membrane integrity or integrin expression [44].

Table IV
LD_{50}s (mJ/cm^2) of HB4a and HB4a-Ras exposed to PDT employing different PSs.

	PII	MC540	AO	Verteporfin	m-THPC	Ce6
$HB4a_{adh}$	23	73	17	6.2	15	12.5
$HB4a_{susp}$	110	406	62	37	54	58
$HB4a\text{-}Ras_{adh}$	41	120	22	12.2	27	32
$HB4a\text{-}Ras_{susp}$	400	>700	491	110	216	250

In the present work, we have shown that some parameters related to adhesion are altered in Ras-overexpressing cells. Vinculin –a protein involved in adhesion to the substrate– is equally expressed in both parental and transfected cells but its subcellular distribution is different. Similarly, actin shows the same expression, but its distribution is different between the two cell lines, thus showing that the distribution of actin cytoskeleton proteins is affected by Ras. The different distribution of vinculin and actin within the cells can lead to modifications in their function, mainly those related to cell motility and adhesion.

The protein E-cadherin involved in cell-cell adhesion is lower expressed in HB4a-Ras transfected cells as compared to wt cells, its distribution is aberrant after the oncogene activation, displaying a pattern of interdigitations instead of a classical arrangement in adherence junctions.

In previous work, we have found redistribution of β-actin, E-cadherin and vinculin in murine tumour mammary cells resistant to ALA-PDT [29]. Breast and other cancers which have up-regulation of Ras often display down-regulation or mislocalisation of E-cadherin [45]. Ras transformation has been suggested to disrupt E-cadherin junctions, causing both mislocalisation of E-cadherin away from the cell surface together with decreased expression [46].

Cell-cell and cell-substratum reduction of adhesion associated with tumour progression were related to cytoskeleton proteins, particularly actin and vinculin, and to the loss of ECM proteins such as fibronectin [46,47]. These arrangements can be induced by oncogenic activation [48]. In addition, adhesion molecules are overexpressed in some drug-resistant cells [49].

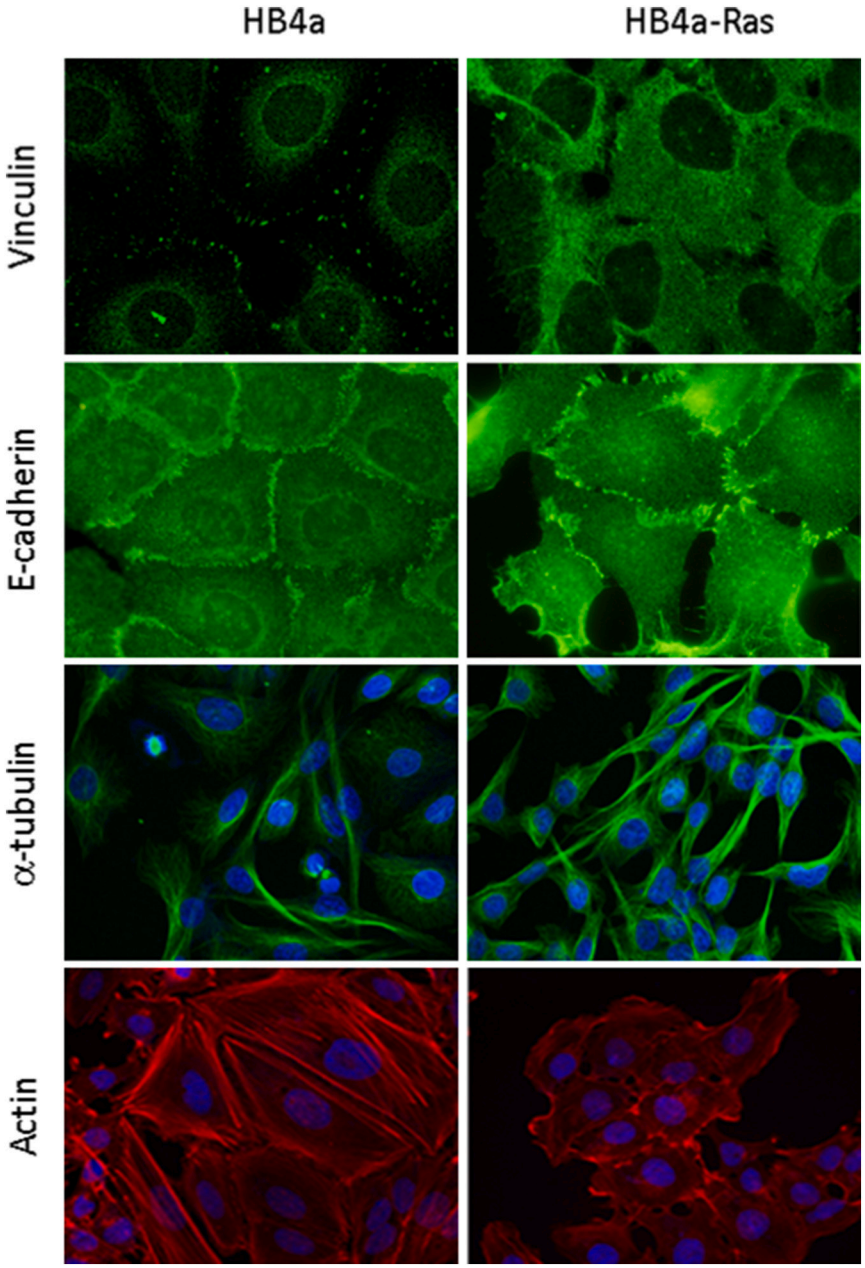

Fig. 5. Western blot analysis of E-cadherin, vinculin and β1 integrin of HB4a and HB4a-Ras cells.
Total extracts of subconfluent cultures were employed. Tubulin and actin were employed as loading controls. Figures are representative of at least three independent experiments. Quantification of bands was obtained from triplicate samples and normalized onto β-actin or α-tubulin is represented in the bars graph; * two-tailed unpaired Student's *t*-test.

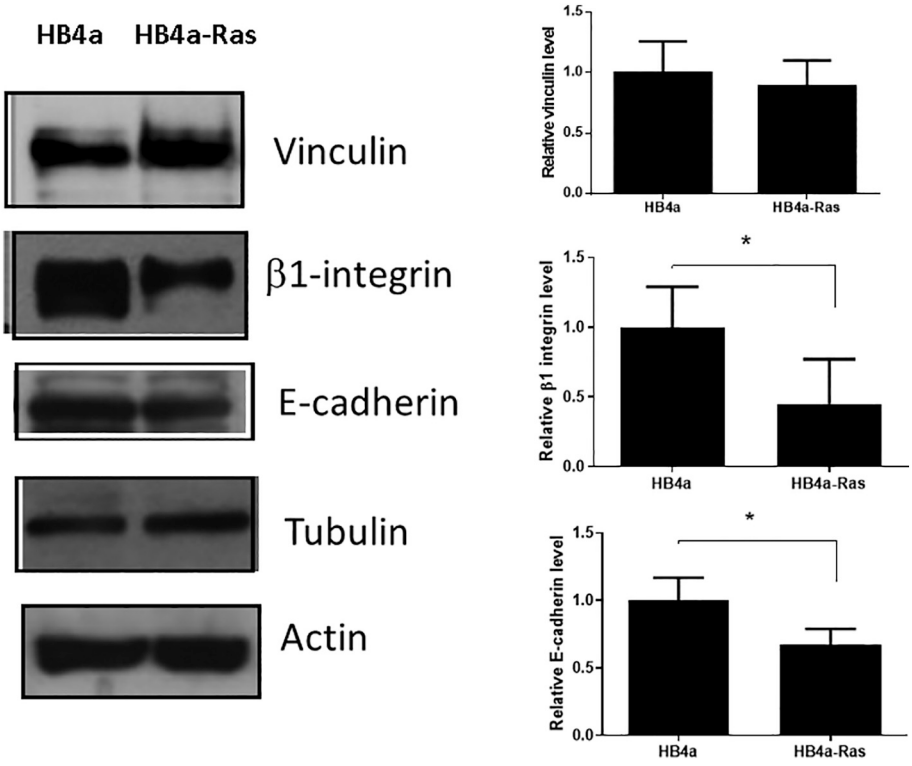

Fig. 6. Expression pattern of β-actin, α-tubulin, E-cadherin and vinculin in HB4a and HB4a-Ras cells.
Magnification 40× and DAPI counterstaining for β-actin and tubulin, and magnification 63× for vinculin and E-cadherin. Figures are representative of at least three independent experiments.

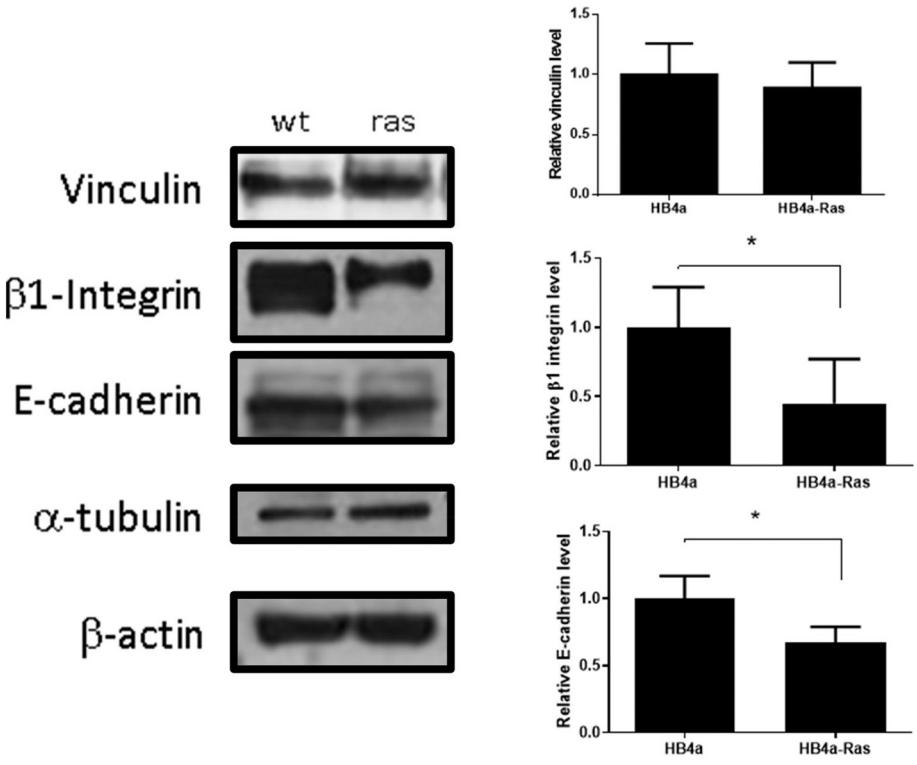

Fig. 7. Adhesion of HB4a and HB4a-Ras cells to ECM proteins.
An adhesion assay was carried out letting the cells attach to 96-well plates coated with concentrations of 1 to 5 μg/ml of fibronectin, 5 to 20 μg/ml of collagen I and IV and vitronectin and 10 to 30 μg/ml of laminin. Data represent the mean ± SD for three independent experiments performed in quadruplicates $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ between HB4a and HB4a-Ras, ANOVA followed by Tukey's test.

Moreover, adhesion of HB4a-Ras cells to ECM proteins fibronectin, collagen I and IV and laminin, is impaired as compared to its normal counterpart, but not to vitronectin. Unspecific adhesion to BSA is diminished as well in the oncogene-transfected cells. In addition, whereas HB4a cells adhere to all ECM proteins studied in a substrate concentration-dependent manner, HB4a-Ras cells show concentration

dependence only in adhesion to fibronectin, vitronectin and slightly to laminin.

Vitronectin interacts mainly with β3 and β5 integrin subunits. Integrin αvβ3 –also known as vitronectin receptor– plays a crucial role in tumour progression [56]. However, β3 integrin is not expressed by HB4a or HB4a-Ras cells. Since β1 is downregulated in HB4a cells after Ras insertion, we hypothesize that this integrin may be involved in the binding to fibronectin, collagen I and IV and laminin but not of vitronectin in HB4a-Ras cells.

H-Ras can either suppress or activate integrins, depending on the cellular context and the type of integrin it affects [14,50–52]. This cell-type dependence of H-Ras-mediated integrin regulation exemplifies the intricacy of intracellular signalling [14].

We have shown that when cells are maintained under conditions of suspension growth, the PDT effect is impaired significantly as compared to adhesive cells. Moreover, in cells overexpressing Ras, which are less adherent *per se*, the resistance conferred by the suspension conditions is more marked as compared to the parental cells.

On the one hand, we believe that the increased resistance to PDT mediated by Photofrin found in both HB4a and HB4a-Ras cells under suspension conditions could be attributed to the fact that floating cells are not reached by light to the same extent that cells attached to the surface. On the other hand, the more marked gain of resistance to PDT observed in the suspended Ras-transfected cells, accounts for the higher ability conferred by Ras to survive in conditions of decreased cell-ECM interactions.

Even when Ras-transfected cells present lower adherence to the substratum, this does not make them more sensitive to drugs or other injuries. On the contrary, adhesion-regulated programmed cell death (ARPCD) can be substantially suppressed by the expression of H-RAS, as has been observed previously [55]. RAS oncogenes may contribute to tumourigenesis by indirect promotion of cell survival and three-dimensional growth in certain solid tumours. An activated form of DRAS rescues the loss of integrin-dependent anoikis in the wing imaginal disc of *Drosophila* [56].

Ras signalling involves a complex arrangement of pathways, consisting of cross-talk, feedback loops, branch points and multi-component signalling complexes. Ras activation is not the type of signalling pathway induced by one specific ligand but contributes to signalling induced by multiple factors controlling cellular responses [53,54]. Recently, how Ras oncogenes alter cell mechanics in epithelial tissues has been reviewed, highlighting the multiple targets involved in which Ras alters the ability of cells to sense the stiffness of their environment through changes to cell contractility and substrate adhesion [55].

5. Conclusions

To sum up, in the present study we found that oncogenic activation of Ras is capable of conferring resistance to photodynamic treatment independently of the nature and intracellular accumulation of the PS (except for AO). This resistance appears to be related at least to some extent to differences in adhesion, actin cytoskeleton proteins distribution, and adhesion to ECM, although due to the complexity of the multiple signalling pathways involved in Ras activation, many other factors could be involved.

Knowledge of the mechanisms of resistance to photodamage in Ras-overexpressing cells may lead to the optimization of the combination of PDT with other modalities of treatment, although further studies are needed to extrapolate our observation to other cells overexpressing Ras.

CRediT authorship contribution statement

Lorena Rodriguez: Investigation, Formal analysis. **Gabriela Di Venosa:** Investigation, Formal analysis. **Martín A. Rivas:** Investigation, Writing – original draft. **Angeles Juarranz:** Supervision, Funding acquisition. **Francisco Sanz-Rodriguez:** Supervision, Funding acquisition. **Adriana Casas:** Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All of the data supporting our findings can be found in the main paper and Supplementary material. Data are however available from the authors upon reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.121287.

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