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Small Interfering RNA-Mediated Silencing of Glutathione-*S*-Transferase A1 Sensitizes Hepatic Carcinoma Cells to Photodynamic Therapy with Pentaphyrins

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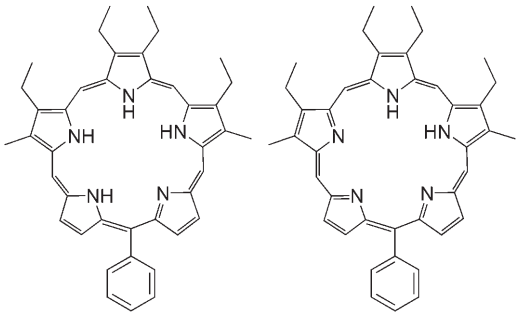
Photodynamic therapy (PDT) is a relatively new clinical treatment applied to a variety of human tumors.[1] This technique involves the administration of a photosensitizer devoid of mutagenic properties, and exposure of the pathological area to visible light to produce cytotoxic reactive oxygen species (ROS), which kill malignant cells by apoptosis and/or necrosis. An important goal in medicinal chemistry, pursued in many laboratories, is the search for new photosensitizers that absorb at wavelengths >630 nm, causing low generalized sensitivity effects and that are characterized by enhanced tumor specificity. A considerable effort has been devoted to expanded porphyrins, which have a larger polypyrrolic macrocycle than normal porphyrins. The most studied expanded porphyrin is sapphyrin, which contains a macrocycle with five pyrroles and five *meso*-carbons.[2] This molecule has interesting properties, including the capacity to complex anions,[3] to produce relatively high yields of singlet oxygen, and to absorb light in the near-infrared region.[2] We recently synthesized two expanded polypyrrolic macrocycles: nonaromatic 24-π-electron isopentaphyrin **1** and aromatic 22-π-electron porphyrin **2**, each with five pyrroles in the macrocycle and five *meso*-carbons. The aromatic and nonaromatic pentaphyrins are non-cytotoxic in the dark, but upon irradiation with visible light they promote a strong photodynamic effect in a number of cell lines, with IC_{50} values in the range of 1–4 μM upon exposure to a light fluence of 8 mW cm^{-2}.[4] Pentaphyrins **1** and **2**, after activation with light, promote cell death by apoptosis as suggested by cell cytometry and caspase-3/7 assays.[4]



1 **2**

As the hallmark of PDT is the generation of oxidative stress (singlet oxygen, hydroxyl radical, and superoxide anion) by the activated photosensitizer, the efficacy of PDT depends on the balance between the cellular defenses against oxygen toxicity and the generation of ROS. Under oxidative stress cells normally increase the levels of detoxification enzymes including superoxide dismutase, catalase, glutathione peroxidase and glutathione-*S*-transferases.[5] One working hypothesis couples PDT with the antioxidant systems of the cell and suggests that by silencing the genes that encode the detoxification enzymes, the cells should be sensitized to PDT. To provide a proof-of-concept, we performed experiments using a hepatic cell line (HepG2) because under oxidative stress, glutathione-*S*-transferase α1-1 (GSTA1-1) is overexpressed in the liver.[6] The glutathione transferases (GSTs, EC 2.5.1.18) are detoxifying enzymes that catalyze nucleophilic attack by reduced glutathione on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulfur atom. Three major protein families that are widely distributed in nature exhibit glutathione transferase activity. Two of these, cytosolic and mitochondrial GST, comprise soluble enzymes that are only distantly related. The third is a membrane-associated GST (microsomal enzyme), and is now referred to as MAPEG enzyme. Based on amino acid sequence similarities, seven classes of cytosolic GST are recognized in mammalian species: α, μ, σ, θ, ω, and ζ.[7] Cytosolic GSTs including GSTA1-1 are often overexpressed in tumor cells.[8] Owing to its detoxifying activity against xenobiotics and ROS, GSTA1-1 represents an important cellular response to oxidative stress, activating a number of molecular mechanisms including: 1) degradation of lipid peroxides, 2) binding of pro-oxidant compounds such as photosensitizers, and 3) modulation of signal transduction, preventing apoptosis.[9] On this ground, we reasoned that silencing or at least down-regulating GSTA1-1 should result in a weaker cellular response to oxidative stress, with the consequence that a photosensitizer should promote a stronger photokilling.

In order to specifically silence GSTA1-1 expression in hepatic carcinoma HepG2 cells, we used small interfering RNA (siRNA) technology.[10] On the basis of the *GSTA1-1* nucleotide sequence (NM_145740), three 21-base-pair siRNA duplexes were designed to attempt the silencing of this gene.[11] The activity of the designed 21-mer siRNAs were tested by transfecting HepG2 cells and measuring the level of GSTA1-1 protein in the treated and untreated cells. The siRNA showing the highest

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GSTA1-1 inhibitory activity was cloned in plasmid pSUPER[12] to obtain an expression vector called pGST1, which directed the endogenous synthesis of an siRNA-like transcript. The primary transcript was designed in such a way that it folds back on itself to generate a 19-base-pair stem–loop structure, which is then transformed into the active siRNA. As a control, we constructed a vector, pGSTc, that expresses an siRNA containing one C/G inversion (Figure 1). HepG2 cells were transfected

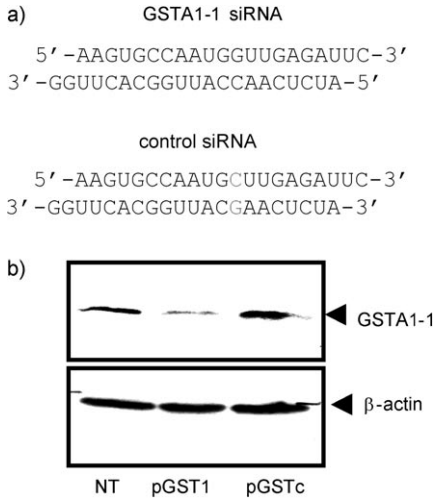

Figure 1. a) siRNA sequences produced by pSUPER. b) Western blot carried out 72 h following transfection, showing that the level of GSTA1-1 protein is ~50% of the level measured in non-treated cells (NT).

with the two expression vectors by using lipofectamine, and 72 h following transfection, the total protein content was extracted and subjected to Western blot analysis. It can be seen that the level of GSTA1-1 protein in the cells transfected with pGST1 is ~50% of that measured in non-transfected cells or those transfected with control pGSTc. This experiment shows that the designed siRNA vector is able to knock down the target *GSTA1-1* gene. Next, we measured the photokilling effect of pentaphyrin **2** in HepG2 cells transfected with pGST1 or pGSTc (Figure 2). HepG2 cells were transfected with the siRNA vectors (pGST1 or pGSTc) using lipofectamine, treated with pentaphyrin **2**, which was added to the culture medium (at 24 h), irradiated for 30 min with visible light at a fluence of 8 mW cm^{-2} (at 48 h), and the cell viability was measured by the resazurin assay (at 72 h). Following this protocol, the cell viability assay was performed in HepG2 cells under conditions in which the endogenous level of GSTA1-1 protein was reduced by roughly 50%. The experiments were performed with three doses of pentaphyrin **2**: 3, 5, and 7.5 μM. Figure 2 shows the results of a typical experiment carried out with 3 μM **2**. Relative to 100 as the viability of non-treated HepG2 cells, it is evident that **2** produced a stronger growth decline (~60%) in cells previously transfected with 0.2 μg siRNA-generating vector pGST1, compared with HepG2 cells treated only with pentaphyrin (~20%). This synergistic effect due to the combined pGST1–pentaphyrin treatment supports the hypothesis of this study: that knocking down the expression of the antioxidant GSTA1-1

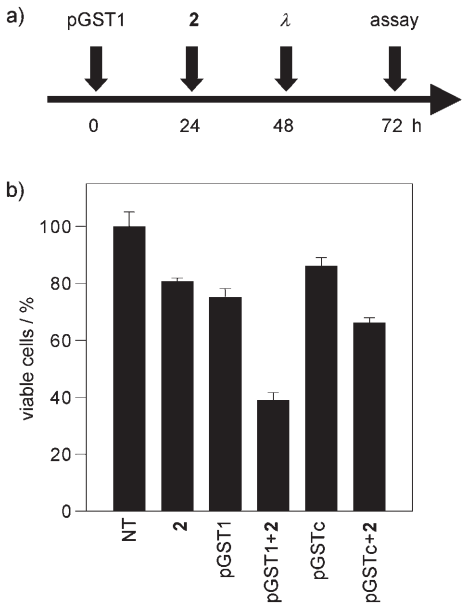

Figure 2. a) Experimental procedure for the transient transfection experiments. b) Percent cell viability relative to wild-type HepG2 cells (NT) in groups treated with pentaphyrin **2**, siRNA vectors pGST1 and pGSTc (control), and combined treatment with **2** + vectors. Pentaphyrin **2** was used at a concentration of 3 μM; vectors (0.2 μg) were transfected with lipofectamine, and the cells were irradiated for 60 min at a fluence of 8 mW cm^{-2}.

should sensitize malignant cells to PDT. Notably, **2** does not produce any synergistic effect in HepG2 cells transfected with control pGSTc. The data obtained with the three pentaphyrin concentrations are shown in Table 1.

Table 1. Photokilling in HepG2 cells treated with increasing amounts of pentaphyrin **2**, siRNA vectors + **2**, and siRNA vectors alone.

[**2**] [μM]	**2** [%][a]	**2** + pGST1 [%][b]	Combined effect [%][c]	pGST1 [%][d]	pGSTc [%][e]
3	19 ± 1	61 ± 5	42	25 ± 3	15 ± 4
5	49 ± 4	73 ± 4	24	17 ± 3	10 ± 2
7.5	61 ± 3	72 ± 4	11	15 ± 3	8 ± 0.2

[a] Percent photokilling in cells treated with pentaphyrin **2** (concentrations indicated in leftmost column). [b] Percent photokilling in cells transfected with pGST1 and treated with pentaphyrin **2**. [c] Synergic effect due to the combined treatment with pGST1 + **2**. [d,e] Percent photokilling in cells transfected with pGST1 or pGSTc alone.

Comparing the percent photokilling observed in the cells treated with only pentaphyrin (column 2, Table 1) with that obtained with pentaphyrin–siRNA treatment (column 3), one observes that the combined treatment is more efficient with incremental photokilling, varying from 42 to 11%. Furthermore, the same amount of photokilling (~70%) caused by a high dose of photosensitizer (7.5 μM) is obtained with a lower photosensitizer dose (3 μM) if the cells are treated with the siRNA vectors. This finding has an important practical consequence for PDT, because the combined treatment can avoid high pho-

tosensitizer doses, which normally cause undesirable generalized photosensitivity effects.

We next addressed the question of whether a genetically modified cell line, in which siRNA specific for *GSTA1-1* is stably expressed, is more sensitive to PDT. To stably introduce a conditional knock-down system specific for *GSTA1-1* in HepG2 cells, we followed the method previously described by van de Wetering et al.[13] Two vectors, appropriately engineered, were stably integrated in the HepG2 genome (Supporting Information). The resultant HepG2 clone contained an inducible system that allowed the endogenous production of siRNA molecules upon cell treatment with doxycycline. Figure 3a shows

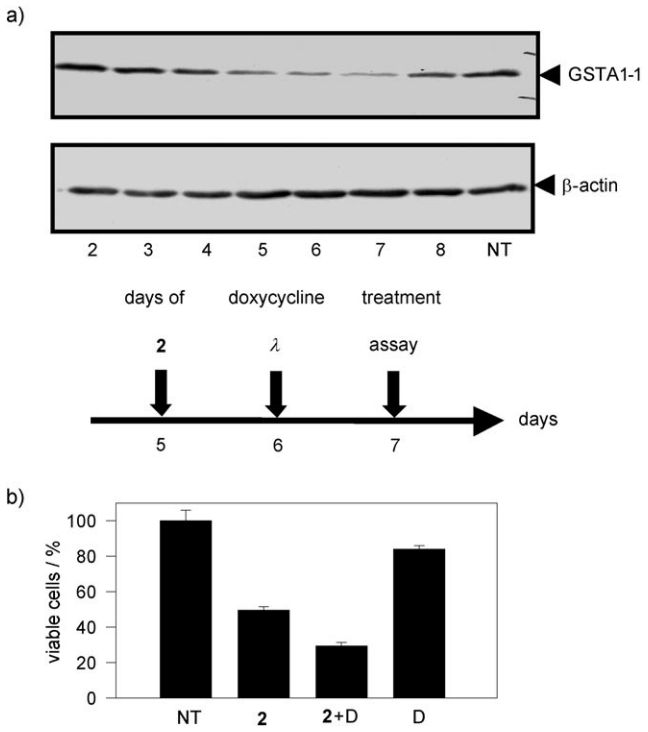

Figure 3. a) Western blot analysis showing progressive suppression of GSTA1-1 protein expression in the stable HepG2 clone induced by repeated treatment with doxycycline (5 μg mL^{-1}). b) Percent cell viability relative to clone non-treated with doxycycline (NT) determined by resazurin assays in the clone treated with **2**, clone treated with **2** + doxycycline (**2** + D), and clone treated with doxycycline alone (D).

a progressive decrease in the expression of GSTA1-1 protein, but not of β-actin, in the transformed HepG2 cells after addition of the antibiotic to the culture medium. The maximum inhibition (~60%) was observed after 7 days of doxycycline treatment. The level of GSTA1-1 recovered after longer periods, probably because cells expressed multidrug-resistance proteins, thus decreasing the intracellular level of doxycycline.[14] In addition to the stable clone generating siRNA specific for *GSTA1-1*, we constructed a second stable clone as a control, which produces siRNA molecules bearing seven mutations relative to the wild-type siRNA (Supporting Information). The two stable clones were exposed to doxycycline for 5 days to induce the suppression of the GSTA1-1 protein. They were then treated with 5 μM pentaphyrin **2** and subsequently irradiated for 60 min at a fluence of 8 mW cm^{-2}. The percent cell viability was measured by the resazurin assay, and the results are reported as histograms in Figure 3b. It can be seen that the photokilling in the stable HepG2 clone treated with only pentaphyrin reduces cell growth by 51 ± 2% with respect to the untreated clone, whereas the inhibition of cell growth is 71 ± 2% for the clone in which the expression of GSTA1-1 was suppressed by repeated treatment with doxycycline. The treatment with doxycycline alone does not influence cell viability. These data agree with those obtained with the transient transfection experiments and support the validity of combined treatments.

In summary, hepatic HepG2 cells can be sensitized to photodynamic treatment with pentaphyrin by knocking down the antioxidant *GSTA1-1* gene using siRNA. This study provides a proof-of-concept for combining PDT with antigene strategies against genes involved in cellular responses to oxidative stress. It was also found that a combined PDT–siRNA treatment requires a lower photosensitizer dose to obtain the same photokilling effect than with photosensitizer alone. This observation may have important practical consequences in PDT, as undesirable generalized photosensitivity side effects increase with photosensitizer concentration. Detailed experiments to assess the photokilling effect of knocking down other antioxidant genes, either individually or in combination, are underway.

Experimental Section

Cells and culture conditions. HepG2 cells were grown in DMEM/high glucose (Celbio, Milan, Italy) with penicillin/streptomycin (100 mg mL^{-1}), 2 mM glutamine, and 10% fetal bovine serum (heat inactivated at 56 °C for 20 min) at 37 °C in 5% CO_2. Cells were subcultured twice per week to keep them in log phase.

Transient clone. HepG2 cells were transfected with pSUPER, containing either the small hairpin RNA (shRNA) GST1 or GSTc sequence, using Lipofectamine 2000 (Invitrogen, Milan, Italy) according to the manufacturer's instructions.

Inducible stable clones. HepG2 cells were transfected with two plasmids, pcDNA6TR and pTer, to produce the repressor and the GSTA1-1-specific shRNA, respectively, as reported by van de Wetering.[13] The clones were grown in a medium supplemented with blasticidin S·HCl (2 μg mL^{-1}, Invitrogen) and zeocin (50 μg mL^{-1}, Invitrogen). Conditional expression of shRNA was induced by the addition of doxycycline·HCl (Sigma) at a concentration of 5 μg mL^{-1}. The pentaphyrin **2** (5 μM) was added to the culture medium.

Western blotting. Total protein lysate (20 μg) was run in 12% SDS–PAGE and blotted onto a nitrocellulose membrane. The membrane was incubated with rabbit polyclonal antibody against GSTA1-1 (1:1000, Calbiochem) or mouse monoclonal antibody against β-actin (1:5000, Calbiochem). Subsequently, it was treated with anti-rabbit IgG (1:5000, Calbiochem) and anti-mouse IgM (1:5000, Calbiochem), respectively. Immunoanalyses were visualized as previously described,[15] and protein levels were quantified by Image Quant TL version 2003 software (Amersham).

Determination of cell proliferation. HepG2 cells (wild-type or stable transfectants) were plated at density of 6000 cells per well in a 96-well plate and allowed to grow according to the scheme reported in the text. The percent viable cells was determined by

the resazurin assay following the manufacturer's instructions (Sigma).

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