Photochemical Internalization of Therapeutic Macromolecular Agents: A Novel Strategy to Kill Multidrug-Resistant Cancer Cells

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ABSTRACT

Drug resistance is a major problem for chemotherapy. Entrapment of anticancer drugs in endolysosomal compartments or active extrusions by plasma membrane proteins of the ATP-binding cassette (ABC) superfamily are important resistance mechanisms. This study evaluated photochemical internalization (PCI) of membrane-impermeable macromolecules that are not the target of ABC drug pumps for treating multidrug-resistant (MDR) cancer cells. We used the drug-sensitive uterine fibrosarcoma cell line MES-SA and its MDR, P-glycoprotein (P-gp)-overexpressing derivative MES-SA/Dx5 with the photosensitizer disulfonated meso-tetraphenylporphine (TPPS2a) and broad spectrum illumination. The PCI of doxorubicin, the ribosome-inactivating protein gelonin and adenoviral transduction were assessed in both cell lines, together with the uptake and excretion of TPPS2a and of two fluid phase markers easily detectable by fluorescence [lucifer yellow (LY) and fluorescein isothiocyanate (FITC)-dextran], as a model of gelonin uptake. Both cell lines were resistant to PCI of doxorubicin, but equally sensitive to PCI of gelonin, even though the endocytosis rates of LY and FITC-dextran were significantly lower in the MDR cells. In control studies, MES-SA/Dx5 cells were more resistant to photodynamic therapy (TPPS2a / light only). This was not mediated by P-gp, as there were no differences in the uptake and efflux of TPPS2a between the cell lines. After adenoviral infection, PCI enhanced gene delivery in both cell lines. In conclusion, PCI of macromolecular therapeutic agents that are not targets of P-gp is a novel therapeutic strategy to kill MDR cancer cells.

Drug resistance results from a variety of both genetic and epigenetic factors and is a major barrier to successful cancer chemotherapy and macromolecular based drug delivery. The most common drug resistance mechanism is multidrug resistance (MDR), which is characterized by simultaneous resistance to various functionally and structurally divergent drugs. MDR is mediated by protein members of the ATP-binding cassette (ABC) superfamily, in which overexpression of P-glycoprotein (P-gp) is the best-characterized. P-gp functions as a unidirectional efflux pump, where drugs are extruded from the cytosol of cells (Gottesman et al., 2002). Drug resistance may also be a consequence of reduced endocytosis or influx of cytotoxic drugs (Shen et al., 1998) or increased secretion as a result of an elevated exocytosis rate (Warren et al., 1991). In addition, entrapment and degradation within the lumen of endolysosomal vesicles is an important obstacle for macromolecular-based cancer therapeutics (e.g., immunotoxins) (McGrath et al., 2003). This has also been reported to be a significant problem both for nonviral and adenovirus-based gene delivery (Hogset et al., 2004). Sequestering of weak base chemotherapeutics in acidic vesicles has also been documented to cause lowered therapeutic efficacy (Altan et al., 1998). Furthermore, defects in apoptotic signaling (Pommier et al., 2004), increased DNA repair (Chu, 1994), and the cytochrome P-450 detoxifying systems (Schuetz et al., 1996) or glutathione (Fojo and Bates, 2003) may also play important roles in the development of drug resistance. MDR reversal in clinical settings with second- and third-generation...
modulators designed to overcome resistance has proven to be very difficult. Although these modulators increase the in vitro cytotoxicity of different neoplastic agents, e.g., doxorubicin (DOX) and also their plasma concentration in vivo, they failed to significantly increase the therapeutic index (Donnenberg and Donnenberg, 2005). Consequently, the development of methods that can enhance cellular delivery of anticancer drugs and bypass the drug resistance mechanisms of the targeted malignant cells is crucial.

Photochemical internalization (PCI) is a drug and gene delivery method recently developed to improve the release of macromolecules and hydrophilic chemotherapeutic agents from endosomes and lysosomes to the cytosol of target cells (Berg et al., 1999; Selbo et al., 2000a; Hogset et al., 2004). PCI is based on the use of endosomal and lysosomal localizing amphiphilic photosensitizers (e.g., disulfonated aluminum phthalocyanine and meso-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings (TPPS$_{2a}$)), which, after activation by light, induce photodynamic reactions resulting in destruction of endocytic vesicle membranes mediated by reactive oxygen species. There is subsequent release of the entrapped drugs into the cytosol (Berg et al., 1999; Selbo et al., 2000a). PCI of numerous macromolecules has been demonstrated in vitro, including the 30-kDa ribosome-inactivating proteins gelonin and saporin (Berg et al., 1999), gelonin- and saporin-based targeting toxins (Selbo et al., 2000b; Weyer-gang et al., 2006), different peptide nucleic acids (Folini et al., 2003; Shiraishi and Nielsen, 2006), and DNA for gene therapeutic purposes both with viral and nonviral vectors (Hogset et al., 2004). Recently it was shown that using photosensitizers localizing to endocytic vesicles enhanced the delivery of adenoviruses to the nucleus after PCI treatment (Engesaeter et al., 2006). PCI of gelonin and bleomycin and PCI-enhanced nonviral gene delivery has also been documented in vivo (Selbo et al., 2001; Berg et al., 2005; Dietze et al., 2005; Nishiyama et al., 2005; Ndyoe et al., 2006). More recently, it was demonstrated that PCI could make the MDR breast cancer cell line MCF-7/ADR almost as sensitive to DOX as the fully sensitive cells, MCF-7, from the same origin by the intracellular release of endosome-lysosome-trapped DOX (Lou et al., 2006). Accumulation of DOX in MCF-7/ADR cells can be explained by the protonation, sequestration, and secretion (PSS) model (Altan et al., 1998), which suggests that weak base drugs are first protonated in acidified organelles of MDR cells, like lysosomes, where they are sequestered.

In the present study, we aimed to evaluate PCI as a method to deliver macromolecular drugs that escape the P-gp activity of MDR cells. PCI of gelonin or adenosin was performed in the MDR cell line MES-SA/Dx5 and its parental cell line MES-SA. It has been shown that the MES-SA/Dx5 cells do not exert a more pronounced pH-dependent drug accumulation and vesicular sequestration than the parental cells (Wang et al., 2000), but they do overexpress P-gp (Chen et al., 1997). Thus, the MES-SA/Dx5 MDR cell line represents an interesting alternative to the MCF-7/ADR PSS model for evaluation of the PCI technology for treatment of MDR cells.

### Materials and Methods

#### Cell Lines

The human MDR uterine sarcoma cell line MES-SA/Dx5 (ATCC number CRL-1977) was established from the parental cell line MES-SA (ATCC number CRL-1976), grown in the presence of increasing concentrations of DOX. Both cell lines were grown and subcultured in McCoy's 5a medium with 10% FCS, penicillin/streptomycin and glutamine (Sigma, St. Louis, MO). Detachment of MES-SA/Dx5 cells for subculturing was performed at room temperature by adding an EDTA solution containing 0.15 g of disodium EDTA, 4.0 g of NaCl, 0.28 g of sodium bicarbonate, 0.5 g of dextrose, and 0.2 g of KCl dissolved in 500 ml of double-distilled water. Subculturing of the MES-SA cells was carried out with a 0.25% (w/v) trypsin-0.53 mM EDTA solution (Sigma). Human embryonic kidney cells (293) expressing the adenosinoviral E1 gene were purchased from Microbio (Toronto, ON, Canada) and grown in Dulbecco's minimal essential medium containing 10% fetal calf serum and 2 mM glutamine. All cell lines were kept at 37°C in a standard humidified tissue culture incubator with 5% CO$_2$.

#### Drugs and Chemicals

Lucifer yellow CH dilithium salt (LY) (molecular mass 457 Da), fluorescein isothiocyanate (FITC)-dextran (molecular mass 40 kDa), and native gelonin (MW = 30 kDa) were purchased from Sigma. LY was dissolved in water to make a stock solution of 50 mM. FITC-dextran was dissolved in water to make a 25 mg/ml stock solution and gelonin was dissolved in PBS to make a 3 mg/ml stock solution. DOX was purchased from Nycomed Pharma AS (Asker, Norway). The photosensitizer TPP$_{2a}$ (LumiTrans) was a generous gift from PCI Biotech ASA (Oslo, Norway). TPP$_{2a}$ was dissolved in dimethyl sulfoxide to make a 0.35 mg/ml stock solution. The purity of the photosensitizer has previously been assessed and showed one peak on high-performance liquid chromatography (Berg et al., 1999). All dyes were stored at −20°C and protected from light until use. Working solutions of all chemicals were made by dilution in McCoy's 5a culture medium.

#### Light Source

Illumination of cells was performed by using the LumiSource (PCI Biotech ASA), a lamp consisting of four 18-W Osram L 18/67 deehight tubes, which deliver blue light with a mean peak at ~435 nm. The irradiance varies ~10% across the illumination area (45 × 17 cm) with an output of 11.5 mW/cm$^2$. The light box is air-cooled during light exposure, which prevents cells from being exposed to hyperthermia and keeps the irradiance stable over time.

#### Photochemical Internalization of Drugs to Enhance Their Cytotoxic Effect

There were two main aims of these experiments: 1) to see whether the effect of DOX on the two cell lines could be enhanced by adding light and the photosensitizer TPP$_{2a}$, and 2) to see whether the effect of gelonin on the two cell lines could be enhanced by adding light and the photosensitizer TPP$_{2a}$. Cells were seeded in 96-well plates (Nunc, Roskilde, Denmark) at $10 \times 10^5$ cells/cm$^2$ and allowed to attach to the substratum for 5 h at 37°C. Preliminary studies of PDT alone (TPPS$_{2a}$ plus light) were undertaken to establish the light dose that gave ~50% kill of each cell type with the chosen concentration of TPP$_{2a}$ (0.2 µg/ml) and incubation time (18 h) and this dose [0.5 J/cm$^2$ (41 s) for MES-SA and 0.9 J/cm$^2$ (61 s) for MES-SA/Dx5 cells] was used for the PCI experiments in which DOX or gelonin was coincubated at different concentrations. For the DOX study, cells were treated in two different ways. 1) Cells were incubated at 37°C for 18 h with (0.2 µg/ml) TPP$_{2a}$ and DOX at concentrations from 0.1 to 10,000 nM. The cells were then washed three times and incubated in drug-free medium for a further 4 h at 37°C before light exposure. 2) Alternatively, cells were incubated with just TPP$_{2a}$ for 18 h and then washed three times with drug-free medium, incubated for a further 4 h with DOX.
at concentrations from 0.1 to 10,000 nM, and subsequently exposed to light. Control cells were treated with DOX but without TPPS2a or light. For gelonin, only coincidence with the photosensitizer over night was performed. In brief, cells were incubated with gelonin (3.0 μg/ml) and TPPS2a (0.2 or 0.05 μg/ml), followed by a further 4-h incubation in drug-free medium before light delivery [light exposure time 0–150 s (0–2.5 cm²)]. Quantitative assessment of cell survival was performed by means of either the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay 2 days after light exposure or a colony-forming assay 10 days after light exposure (only for the gelonin studies), as described below. All handling of cells in the presence of the photosensitizer was carried out under subdued lighting. Control cells received gelonin alone (0.01–300 μg/ml) with no TPPS2a or light. These cells were incubated with gelonin for 18 h and then were washed and incubated in drug-free medium for a further 48 h before MTT assay.

**Cytotoxicity Assessment**

Cell survival was evaluated mainly by the MTT method (tetrazolium dye reduction) 48 h after light exposure. In brief, culture medium was removed, and cells were incubated in medium with 0.25 mg/ml MTT (Sigma) for 1 h at 37°C. The MTT medium was subsequently aspirated off and replaced with 100 μl of 99% dimethyl sulfoxide (Sigma). The 96-well plate was set on a shaker for 10 min before absorbance was measured at 570 nm. Wells without cells incubated with MTT medium only were used for background subtraction. Alternatively, cell survival was assessed by the clonal cell survival method. In brief, 1000 cells were seeded out per well in 6-well plates (~100 cells/cm²; Nunc) and treated as described above. After visible colonies were obtained 10 to 14 days after light exposure, wells were washed once in 0.9% NaCl and then fixed in 96% ethanol for 10 min. Subsequently cells were stained with a saturated solution of methylene blue (Sigma) for 10 min and then dried before manual colony counting. Colonies with >20 cells were classified as a colony and counted.

**Uptake and Excretion of Dyes and Dextran Particles**

Gelonin is a 30-kDa protein toxin that is taken up in cells by endocytosis and mainly by fluid-phase endocytosis (Madan and Ghosh, 1992; Selbo et al., 2000a) but that is not detectable by fluorescence. To characterize the capacity of both MES-SA and MES-SA/Dx5 cells to accumulate and excrete hydrophilic molecules, the fluid-phase endocytosis markers LY and the 40-kDa substance FITC-dextran were used in this study. The latter has a way of mimicking a macromolecule of size comparable with that of the gelonin used in the PCI experiments. This was assessed using two separate techniques. In the first approach, the uptake of each substance at a range of times after administration was measured by cell lysis and subsequent quantification of the concentrations by extraction and fluorescence (for LY and TPPS2a) and by flow cytometry of trypsinized cells (for FITC-dextran). In the second approach, fluorescence was documented by imaging live cells.

**Quantification of Uptake by Fluorescence.** Cells (0.5 × 10⁶) were allowed to attach to the substratum of 35-mm dishes for ~5 h (No. 3001 Falcon; Becton Dickinson, Franklin Lakes, NJ) before incubation with the substances of interest. For TPPS2a, cells were incubated with a solution containing 0.2 μg/ml and after times from 5 min to 18 h, were removed from the culture medium and washed three times with ice-cold PBS, and 1.0 ml of 0.1 M NaOH was added to the dish. After 5 min, the dissolved cells and monomerized TPPS2a were collected and stored protected from light at room temperature until fluorescence measurements.

To evaluate differences in endocytosis and exocytosis rates between MES-SA and MES-SA/Dx5 cells, we used the fluid phase endocytosis marker LY. For LY assessment, cells were incubated with a 0.5 mM solution in McCoy’s medium with 10% FCS for times from 5 min to 18 h. The cells were then placed on ice and washed three times with ice-cold PBS containing 0.15 M NaCl and 0.015 M NaH₂PO₄ at pH 7.4. Thereafter the cells were lysed with 100 μl of 10% SDS for 15 min at room temperature, followed by incubation with 22 μl of DNase I at 1 mg/ml in 1 mM MgCl₂ (Sigma) for an additional 15-min incubation at 37°C. Then 100 μl of 10% SDS was again added to achieve complete solubilization, and the final volume was adjusted to 1 ml with PBS. Samples were then protected from light and stored at room temperature until fluorescence measurements.

In the PCI protocols used in this study, after incubation with TPPS2a for 18 h, the cells were washed three times and incubated in drug-free medium for 4 h to remove the photosensitizer from the plasma membrane before further procedures. In view of this, the exocytosis kinetics of LY and TPPS2a were studied for this “chase” period by culturing the cells for a further period after the 18-h incubation in medium free of LY or TPPS2a. For LY, after the 18-h incubation the cells were first washed three times with medium with 10% FCS and subsequently isolated for quantification after 5 min to 4 h in the LY-free medium. For TPPS2a, the only time cells were studied was after stopping of the incubation. Subsequently, the levels of LY and TPPS2a were measured as described above.

For LY and TPPS2a, fluorescence in the lysed cellular material was measured using a PerkinElmer LS 50-B Luminescence Spectrophotometer (PerkinElmer Life and Analytical Sciences, Norwalk, CT). For LY, this was set at 428 nm for excitation and 535 nm for detection of emission with a long-pass cutoff filter (530 nm) on the emission side. For each time point, fluorescence of LY bound to the plastic in parallel dishes without cells was used for background subtraction. The zero background level of fluorescence was obtained from cells exposed to only LY-free lysates. Furthermore, the fluorescence of cells that were incubated with LY for <5 s matched the baseline level, which confirmed the efficacy of washing. For TPPS2a, the spectrophotometer was set for excitation at 422 nm and detection at 652 nm with the same long-pass cutoff filter (530 nm) on the emission side. To estimate the absolute concentration of dyes, a standard of known concentrations of LY or TPPS2a was added to cell lysates, which gave a fluorescence intensity of ~50% of the maximum seen in the experimental cells.

To study the distribution of FITC-dextran, cells were incubated with a solution containing 1 mg/ml FITC-dextran. Only two time points were studied: 18 h of incubation and 18 h of incubation followed by three washes with medium containing 10% FCS with a further 4-h incubation in medium free of FITC-dextran. After incubation (18 h or 18 h + 1-h chase in drug-free medium), the cells were washed three times with ice-cold PBS, trypsinized, and diluted in 1 ml of culture medium. Subsequently, cells were passed through a 30-μm filter to exclude aggregates and then immediately cooled and kept on ice in darkness until analyzed by flow cytometry on the FACS Calibur flow cytometer (BD Bisciences) using CELLQuest software.

**Intracellular Localization of TPPS2a, LY, and FITC-Dextran.** Epifluorescence microscopy of live cells was used to analyze differences in the intracellular localization of TPPS2a, LY, and FITC-dextran between the MDR cells and the parental cell line. Only two time points were studied. MES-SA and MES-SA/Dx5 cells (10 × 10⁵ cells/cm²) were incubated for 18 h with 0.2 μg/ml TPPS2a, 0.5 μM LY, or 1.0 mg/ml FITC-dextran. The cultures were examined at this time point or after three washes with medium containing 10% FCS followed by a further 4-h incubation in medium free of the substance of interest. Phase-contrast and fluorescence microscopy were undertaken using a Zeiss Axiosplan epifluorescence and phase-contrast microscope with a 63× oil immersion objective (Zeiss, Oberkochen, Germany), equipped with an air-cooled charge-coupled device camera (Quantix; Photometrics, Tucson, AZ) for acquiring digital phase-contrast and fluorescence photomicrographs. The images were evaluated using an image analysis software program (analySIS PRO 3.0; Soft Imaging System GmbH, Münster, Germany). A HBO/100 W mercury lamp was used for fluorescence excitation. For TPPS2a,
excitation was through a 395 to 440 nm band pass filter with a 470-nm dichroic mirror and a 610-nm long-pass emission filter for recording fluorescence. For cells incubated with LY or FITC-dextran, the excitation filter band width was 450 to 490 nm with a 510-nm dichroic mirror and a 610-nm long-pass emission filter (for LY) and a 510 to 550 band pass emission filter (for FITC-dextran).

**Virus Studies**

**Adenovirus Preparation.** The E1 and E3 deleted adenovirus serotype 5 (Ad5) vector Ad5CMV-lacZ encoding the *Echerichia coli* lacZ gene controlled by the human cytomegalovirus (CMV) promoter was plaque-purified, grown to high titer in 293 cells and purified by CsCl banding (Hitt et al., 1995). Titration of plaque-forming units was performed by plaque assay, and quantification of physical particles was accomplished spectrometrically using a conversion factor of 1.1 × 10¹² viral particles per absorbance unit at 260 nm (Hitt et al., 1995). The ratio of viral plaques to plaque-forming units was 50.

Before infection of cells, the Ad5 was diluted to the desired viral dose in PBS (pH 7.4) containing 0.15 M NaCl, 0.015 M NaH₂PO₄, 0.68 mM CaCl₂, and 0.5 mM MgCl₂.

**Photochemical Internalization of Adenovirus.** Cells were seeded in 12-well plates (Nunc) at 18.4 × 10⁵ cells/cm² and incubated overnight with medium containing 0.2 µg/ml TPPS₂a. The cells were washed three times, and incubated for another 3 h at 37°C in complete medium without TPPS₂a. The medium was then removed, and the cells were incubated with adenovirus in PBS (pH 7.4) containing 0.15 M NaCl, 0.015 M NaH₂PO₄, 0.68 mM CaCl₂, and 0.5 mM MgCl₂. The samples were infected with 5 plaque-forming units [multiplicity of infection (m.o.i.) 5] or 50 plaque forming units (m.o.i. 50) per cell. After 30 min of infection at 37°C, the cells were washed and incubated for another 30 min at 37°C. Then the cells were exposed to 0 to 1500 nM versus 15 nM, respectively), in agreement with a previous study (Harker and Sikic, 1985). However, no PCI-induced increase in DOX sensitivity could be observed in either cell line with the light before or after the technique. The results may even indicate an antagonistic effect of combining the photochemical and DOX treatment in both cell lines. This was also indicated by experiments with a fixed DOX concentration and varying the light dose (data not shown).

The results of the experiments with gelonin are shown in Fig. 1. In the control studies with gelonin alone (Fig. 1A), the MDR MES-SA/Dx5 cells were more sensitive than the parental MES-SA cells at high gelonin concentrations (>30 µg/ml), i.e., ~2.5-fold at IC₉₀ (P = 0.022 for 100 µg/ml gelonin and P = 0.013 for 300 µg/ml gelonin).

The surviving fractions of MES-SA or MES-SA/Dx5 cells subjected to PDT (no gelonin) or PCI of gelonin (3.0 µg/ml), as measured by the MTT assay are shown in Fig. 1, B and C, and as measured by colony counting in Fig. 1D, as a function of the delivered light dose. The PCI groups showed a strong synergistic effect compared with the PDT or gelonin-alone groups. The clonal cell survival results (Fig. 1D) confirmed the MTT results. The cytotoxicity of PCI of gelonin was slightly greater on the MDR MES-SA/Dx5 cells than on the parental MES-SA cells (Fig. 1B), although less noticeable with the lower dose of TPPS₂a (0.05 µg/ml) at which a significant difference (P = 0.033) in toxicity after PCI was achieved only for the highest light dose (2 J/cm²/150 s) (Fig. 1C). It was also of note that a 70% higher PDT dose was needed to reach D₉₀ in Dx5 cells than in the MES-SA cells. Thus, although the MES-SA/Dx5 cells are, at the highest light dose (2 J/cm²/150 s), 14 times less sensitive to PDT than the MES-SA cells (P = 0.0046) (Fig. 1B), the two cell lines are equally sensitive to PCI of gelonin at the lower light doses. At higher light doses the MES-SA/Dx5 cells are more sensitive to PCI of gelonin than the MES-SA cells (Fig. 1, C and D). However, there is an indication that the MDR cells are more sensitive than the parental cells to PCI of gelonin at low light doses when a higher TPPS₂a concentration is used (P = 0.001 at 0.4 J/cm²/30 s of light) (Fig. 1B).

**Uptake and Excretion of Dyes and Dextran Particles.** Results of the uptake and excretion studies of TPSP₂a, LY, and FITC-dextran measured by quantitative fluorescence are shown in Fig. 2. There was a rapid uptake of TPSP₂a in the first 2 h (1.5 ng/mg protein/min in both cell lines), which subsequently decreased substantially with no significant differences in accumulated TPSP₂a between the cell lines after 18 h of incubation (Fig. 2A) (P = 0.17). Likewise, there was no difference between the cell lines in the excretion of TPSP₂a as measured after 4 h of drug-free incubation (45 and 50% excretion in MES-SA/Dx5 and MES-SA cells, respectively, P = 0.34) (Fig. 2B), although at both 18 h and after the 4-h washout, the TPSP₂a levels were slightly lower in the MES-SA/Dx5 cells. LY uptake was rapid in the first 15 min in both cell lines (~3.3 nmol LY/mg protein/min), then slowed more in the MES-SA/Dx5 cells. After 18 h the concentration of LY in MES-SA cells was double that in the MES-SA/Dx5 cells (Fig. 2C). During the subsequent washout period of culture in medium free of LY, there was a rapid (~10 min) 30% drop in cell-bound LY in the MES-SA cells that was not observed in the MES-SA/Dx5 cells (Fig. 2D) although by 4 h, the amount of LY had decreased by 50% in both cell
After 18 h of incubation, the MES-SA/Dx5 cells accumulated 40% less FITC-dextran than the MES-SA cells. After the 4-h chase in drug-free medium, the level of FITC-dextran fell by 36 and 23% in the MES-SA/Dx5 and MES-SA cells, respectively. At this time point, the FITC-dextran concentration was 44% lower in the resistant cells (Fig. 2E) ($P_{/H11005}_{/H11005} 0.046$).

Intracellular Localization of TPPS$_{2a}$, LY, and FITC-Dextran. After 18 h of incubation with TPPS$_{2a}$, strong, granular, intracellular fluorescence was seen, together with a weak and diffuse fluorescence pattern suggesting additional plasma membrane localization similar for both cell lines (data not shown). After the 4-h incubation in drug-free medium, the diffuse fluorescence was markedly reduced but with no apparent change in the granular fluorescence. The images obtained after incubation of MES-SA cells with LY also exhibited strong, granular, intracellular fluorescence, thought to represent endocytic vesicles, although the diffuse fluorescence seen with TPPS$_{2a}$ was much less apparent with LY. This was also the case for the MDR MES-SA/Dx5 cells, although the overall fluorescence intensity was lower than that for the MES-SA cells, confirming the quantitative assessments described above. The 4-h incubation in drug-free medium resulted in a reduction of fluorescence in both cell lines. At this time point the fluorescence intensity in the MES-SA/Dx5 cells was markedly lower than that in the MES-SA cells, which is in accordance with the quantitative assessments. Both cell lines displayed a bright granular fluorescence from FITC-dextran, resembling the fluorescence pattern of LY and TPPS$_{2a}$, with somewhat higher fluorescence intensity in the MES-SA parental cell line (data not shown).

Photochemical Internalization of Adenovirus. The effect of the PCI treatment on adenoviral transduction of MES-SA and MES-SA/Dx5 cells was studied as a function of the light dose delivered to the cells. Accordingly, TPPS$_{2a}$-treated cells were incubated with Ad5CMV-lacZ at 5 or 50 infectious units per cell and kept in the dark or exposed to 50 to 70 s (MES-SA) or 65 to 85 s (MES-SA/Dx5) of light from LumiSource. The surviving fractions, as measured by the MTT assay, of MES-SA and MES-SA/Dx5 cells subjected to light, were $\sim 60$ to 40%, depending on the light dose (data not shown). The percentage of $\beta$-galactosidase-positive live cells increased with the light dose administrated to the cells for both virus doses tested and in both cell lines (Fig. 3) ($P = 0.023$, analysis of variance). Interestingly, without PCI treatment (although in the presence of TPPS$_{2a}$), adenoviral transduction was more efficient in MES-SA/Dx5 than in MES-SA cells (zero light dose in Fig. 3). Infecting MES-SA/Dx5 cells with a viral dose of m.o.i. 5 enabled a similar level of transduced cells as infecting MES-SA cells with m.o.i. 50. At the lowest dose of adenovirus (m.o.i. 5) PCI induced a moderate increase in the fraction of transduced cells in both cell lines. However, at an m.o.i. of 50 PCI enhanced the fraction of transduced cells substantially, i.e., from 4 to $>50\%$ in the MES-SA cells and from 20 to $>60\%$ in the MES-SA/Dx5 cells.

Discussion

There is a need for new therapeutic methods that avoid the known resistance mechanisms and can be an alternative when resistance has been induced after treatment with es-
tablished therapeutic regimens. The present study indicates that PCI of macromolecular therapeutic agents is a novel method to kill MDR cancer cells. The concept is summarized in Fig. 4.

In a recent study PCI was found to efficiently reverse DOX resistance in the MCF-7/ADR breast cancer cell line, in which PSS is the dominant resistance mechanism (Lou et al., 2006). Here we chose to study the uterine sarcoma MDR cell line MES-SA/Dx5. In contrast with the MCF-7/ADR cells, the MDR MES-SA/Dx5 cells do not accumulate and sequester DOX in a pH-dependent manner (Wang et al., 2000), so this cell line is a suitable model for looking for ways of circumventing a different mechanism of MDR. We evaluated PCI of DOX in MES-SA/Dx5 cells and the drug-sensitive parental line, MES-SA, and, as expected, we were not able to show any synergistic or additive effects in either cell line. Our finding is consistent with the study of Wang et al. (2000), which demonstrated that the parental MES-SA cells accumulate a higher fraction of DOX in the lysosomes compared with the MDR cells.

This study, however, reveals PCI of macromolecular therapeutics as a novel treatment regimen of P-gp-based multidrug-resistant cancer cells, using the plant toxin gelonin and adenovirus as model macromolecules. PCI was found to dramatically enhance the effect of gelonin on both MES-SA and MES-SA/Dx5 cells. Interestingly, the MDR cell line MES-SA/Dx5 was equally or more sensitive to PCI of gelonin than its parental MES-SA cell line. The finding was surprising in view of the results showing that the MES-SA/Dx5 cells are to some extent resistant to PDT, and in addition, that the MDR cells had lower capacity for uptake of LY and FITC-dextran than its parental cells.

To characterize the endocytosis and exocytosis capacities of MES-SA and MES-SA/Dx5 cells, we used the small molecule LY (∼0.5 kDa) and the 40-kDa FITC-dextran as macromolecule model simulating gelonin. By means of quantitative fluorescence analysis, we found that MES-SA cells were able
to accumulate twice as much LY as the MES-SA/Dx5 cells. An additional 4-h chase in drug-free medium resulted in a ~50% decrease of intracellular LY for both of the cell lines, which was in accordance with the microscopy findings. The high excretion rate of LY in the parental MES-SA cells during the first 10 min may indicate a rapid turnover of early endosomes. Therefore, a high fraction of LY that is taken up is likely exocytosed rapidly by the MES-SA cells. We also included 40-kDa FITC-dextran as a macromolecule model for the characterization of the endocytosis and exocytosis capacities of the two cell lines. The parental cells were able to accumulate ~1.7-fold more FITC-dextran than the MDR MES-SA/Dx5 cells after 18 h of incubation. An additional 4-h chase in drug-free medium resulted in decreases of intracellular FITC-dextran by 23 and 36% in the MES-SA and MES-SA/Dx5 cells, respectively, thereby resulting in an ~2-fold higher concentration of FITC-dextran in the parental MES-SA cells. Based on these data, one would expect that the parental cells would be more sensitive to PCI of gelonin because they have a higher drug accumulation capacity. However, it has been reported that drugs accumulate to a higher extent in the MES-SA cells compared with the MES-SA/Dx5 cells (Wang et al., 2000), and therefore, the mechanism behind the increased sensitivity to PCI of gelonin of the MDR cells might be due to less lysosomal degradation of gelonin than in the parental cells. In fact, it was observed that the MES-SA/Dx5 cells were more sensitive to relatively high concentrations of gelonin alone (>30 μg/ml) than the parental cells, indicating less accumulation of gelonin in the lysosomes and thus a slower degradation rate in the MES-SA/Dx5 cells.

There were no statistically significant differences in the rate of either uptake or secretion of TPPS₂₄ between the MES-SA and the MES-SA/Dx5 cells. We therefore suggest that the resistance of the MES-SA/Dx5 cells to PDT cannot be attributed to the overexpression of P-gp, which is in accordance with other studies showing that chlorins and porphyrin-based photosensitizers are not substrates for P-gp (Capella and Capella, 2003). Previously, it was shown that murine leukemia P388/ADR cells were not cross-resistant to mesoporphyrin-PDT (Kessel and Erickson, 1992). However, later it was demonstrated that MDR cells can be cross-resistant to PDT because of impaired cellular accumulation of photosensitizers due to P-gp efflux pump activity (Kessel et al., 1994). On the other hand, it has been demonstrated that meso-tetraphenylporphine-PDT was significantly more cytotoxic against a human breast cancer MDR cell line than its parental cells (Teiten et al., 2001). Based on our findings, we cannot explain why the MES-SA/Dx5 cells are less responsive to PDT. One possibility could be differences in endocytic vesicle localization of TPPS₂₄. As pointed out above the drug transport to lysosomes has been shown to be higher in the MES-SA cells than in the MDR MES-SA/Dx5 cells (Wang et al., 2000). Because the uptake of TPPS₂₄ is similar in both cell lines one may speculate that the lysosomal targeting by PDT induces a stronger cytotoxic effect than PDT of endosomes, because lysosomal membrane rupture by means of PDT is expected to result in release of proapoptotic factors (Reiners et al., 2002).

PCI had a beneficial effect on adenovirus-mediated gene delivery to both cell lines. Of note, when the samples were not subjected to PCI treatment, 10-fold fewer viral particles were necessary to obtain comparable levels of transduction in MES-SA/Dx5 cells compared with MES-SA cells. In contrast, the enhancement in reporter gene expression after photochemical treatment was more pronounced in MES-SA cells than in MES-SA/Dx5 cells. This could be due to different levels of Ad5 receptors and/or cell type-specific modes of intracellular transport pathways between the two cell lines tested. Conventional infection with Ad5 is

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**Fig. 3.** PCI-enhanced adenoviral delivery in MES-SA/Dx5 (○, ■) and MES-SA (○, △) cells. Adenoviral transduction was carried out with 5 or 50 infectious units (m.o.i. 5 or 50). Data represent a typical result. Error bars are mean ± S.D. of triplets.

**Fig. 4.** Principle of PCI of macromolecular drugs in MDR tumor cells. A, P-gp pumps doxorubicin (D) out of the tumor cell, a major obstacle in chemotherapy of cancer. B, the macromolecular drug (M) is taken up together with the photosensitizer (S) by endocytosis. The drugs colocalize and are entrapped in endocytic vesicles, another common drug resistance mechanism. M is subjected to enzymatic degradation in the lysosomes. C, release of M from endosomes or lysosomes to cytosol by PCI. M subsequently reaches its intracellular target (e.g., the target for 30-kDa gelonin is 28 S RNA). M is too large to be effluxed by P-gp (or other ABC transporters).
highly dependent on the expression of the Coxackie- and adeno-virus receptor and αv-integrins on the cell surface (Greber et al., 1993). Thus, if Ad5 endosomol release per se is more efficient in MES-SA/Dx5 cells than in MES-SA cells, this might contribute to the difference in the effect of the photochemical treatment. Cell line-dependent effects of PCI have been demonstrated earlier, both for adeno-virus and adeno-associated virus vectors (Bonsted et al., 2005), indicating that the intracellular adenovirus trafficking pathway may differ between cell lines. Because Coxackie- and adeno-virus receptor expression is often reported to be higher in normal cells, the adenovirus has to be re-targeted, e.g., with a growth factor targeting a certain receptor, to increase the specificity to tumor cells (Bonsted et al., 2006).

Clinically, PCI has great potential and may take advantage of the therapeutic experiences of PDT and macromolecular therapies. PCI is a technology developed from PDT. PDT is approved for several cancer indications as well as age-related macular degeneration (Dolmans et al., 2003). The photosensitizer is delivered systemically and accumulates preferentially in neoplastic lesions (2–3:1 relative to normal surrounding tissue) within 2 to 3 days after administration. The therapeutic effect requires activation by light, which usually is delivered by a laser through optical fibers. The treatment may occur by noninvasive light exposure of the tumor, by exposure to the tumor bed after surgical resection of the bulk tumor material, or by interstitial delivery of the light. Thus, in principle, most solid tumors may be treated with PDT. The experience and light delivery applicators used in PDT can also be used in PCI. Gelonin as used in this study, is not suitable for systemic delivery because of its rapid renal clearance and lack of specificity. Local delivery of gelonin as documented in preclinical models (Selbo et al., 2001; Dietze et al., 2005) and used clinically in brachytherapy of prostate cancer should, however, not be excluded. However, it has also been shown that PCI of targeted macromolecules including gelonin enhances in a synergistic manner the therapeutic potential of the macromolecules. The utilization of PCI of targeted macromolecules may therefore induce 3-fold specificity, i.e., preferential accumulation of the photosensitizer in neoplastic lesions, light-directed activation of the photosensitizer, and the use of therapeutic macromolecules with a targeting moiety. Thus, the present study is encouraging for treatment of MDR tumors and should warrant further development of the technology for clinical use either as a local treatment or based on systemic delivery of targeted macromolecular therapeutics.

In conclusion, we have demonstrated the concept of using PCI of macromolecular therapeutics as an alternative strategy to eradicate MDR cancer cells. Future studies including retargeting of macromolecular therapeutic agents with antibodies or growth factors to increase the selectivity toward MDR tumor cells in vivo is clearly warranted.

References


Selbo PK, Sivam G, Fodstad O, Sandvig K, and Berg K (2001) Intracellular adenovirus trafficking pathway may differ between cell lines. Because Coxackie- and adeno-virus receptor expression is often reported to be higher in normal cells, the adenovirus has to be re-targeted, e.g., with a growth factor targeting a certain receptor, to increase the specificity to tumor cells (Bonsted et al., 2006).

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accumulation of these compounds associated with reduced plasma membrane binding proteins. Cancer Res 56:268–275.


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