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Photodynamic Therapy Targets the mTOR Signaling Network *in Vitro* and *in Vivo*

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Abstract: Mammalian target of rapamycin (mTOR) is a regulator of cell growth and proliferation and its activity is altered in many human cancers. The main objective of this study was to evaluate *in vitro* and *in vivo* targeting of mTOR by photodynamic therapy (PDT), a treatment modality for cancer. The amphiphilic endolysosomal localizing photosensitizer $AlPcS_{2a}$ and the p53 mutated rapamycin-resistant colon adenocarcinoma cell line WiDr were used as models. $AlPcS_{2a}$-PDT downregulated the levels of Ser^{2448} phosphorylated mTOR (p-mTOR), total mTOR and phosphorylation of ribosomal S6 (p-S6) immediately after light exposure in a dose-dependent manner, indicating a direct targeting of the mTOR signaling network. Low-dose PDT attenuated the level of p-mTOR in a transient manner; ~35% reduction of p-mTOR was obtained 5 min after a LD_{35} PDT dose, but returned to the basal level 24 h later. Treatment with the mTOR inhibitor rapamycin reduced the p-mTOR level by 25% after 4−24 h of incubation. Combination treatment of rapamycin and PDT *in vitro* resulted in synergistic cytotoxic effects when rapamycin was administered after PDT. However, antagonistic effects were obtained when rapamycin was incubated both before and after PDT. *In vivo*, activated mTOR in the WiDr-xenografts was downregulated by 35 and 75% 5 min and 24 h post PDT respectively as measured by immunoblotting. In contrast to untreated tumors where p-mTOR expression was found throughout the tumors, immunohistochemical staining revealed only expression of p-mTOR in the rim of the tumor at 24 and 48 h post PDT. In conclusion, $AlPcS_{2a}$-PDT is a novel mTOR-targeted cancer therapy. Rapamycin synergistically enhances the cytotoxicity of PDT only when administered post light exposure.

Keywords: mTOR; S6; photodynamic therapy; rapamycin; cancer therapy

Introduction

The 289 kDa serine/threonine kinase mammalian target of rapamycin (mTOR) located downstream of the PI3K-Akt has emerged as a major regulator of cell growth and a central modulator of cell proliferation, migration, differentiation and survival. The activity of mTOR is regulated by different physiological stimuli such as ATP and nutritional levels, growth factors, and stress stimuli including hypoxia, UV light and ROS.[1] In many human cancers, upstream (PI3K-Akt) and downstream (4E-BP1 and S6 kinase) signaling pathways of mTOR are deregulated, and hence, mTOR has been suggested as a target for suppression of tumor growth.[2,3]

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Two functionally distinct mTOR complexes (mTORC), mTORC1 and mTORC2, have been identified.[4] mTORC1 consists of mTOR, mLST8 and raptor (regulatory associated protein of mTOR) and mTORC2 consists of mTOR, mLST8 and rictor (rapamycin insensitive companion of mTOR). mTORC1 is sensitive to the macrolide antibiotic rapamycin and its synthetic analogues CCI-779, RAD001 and AP23573, all specific inhibitors perturbing mTORC1 activity. mTORC2 was thought to be insensitive to rapamycin; however, recently it was shown that long-time incubation with rapamycin inhibited the assembly of the protein complex.[5] The mechanism of action of rapamycin, and its analogues, has created significant interest in their use as anticancer agents. Rapamycin binds to its intracellular receptor FKB12-binding protein (FKBP) making a complex that binds to mTOR and thereby inhibits activation of downstream targets. Inhibition of mTOR by rapamycin induces G1 arrest or, in some cases, apoptosis.[2,4] However, intrinsic or acquired resistance to rapamycin treatment of cancer cells has been demonstrated.[6] Potent rapamycin analogues (rapalogs) or alternative mTOR targeting strategies are therefore highly warranted.[7,8] In addition, cells with mutated p53 have deregulated mTOR expression.[9]

Photodynamic therapy (PDT) is an approved treatment modality of a range of cancers worldwide and was recently recommended as a first line treatment for non-melanoma skin cancers.[10] PDT is a noninvasive and cancer-selective modality based on local or systemic administration of a tumor-accumulating photosensitizer that is subsequently activated by local light exposure resulting in generation of cytotoxic reactive oxygen species (ROS).[11−13] PDT inactivates cancer cells by oxidation of biomolecules, such as some amino acids, unsaturated fatty acids and cholesterol, which cause stress-induction resulting in necrosis and/or apoptotic cell death.[14,15] Recently, it was shown by independent groups that autophagy is an alternative mode of cell death post PDT.[16,17] However, it has been reported that autophagy can protect cells from phototoxicity post low-dose PDT.[18] PDT-generated antitumor activity can also be achieved by targeting the microvasculature and/or activation of the host immune system.[19] Cellular signaling after PDT is dependent on the physiochemical properties and subsequent intracellular localization of the photosensitizer. In the present work an amphiphilic photosensitizer, $AlPcS_{2a}$, known to localize to endosomes and lysosomes was used.

The main aim of this study was to evaluate PDT as an alternative mTOR-targeting strategy. We here report on the PDT effects on mTOR signaling both *in vivo* and *in vitro* in p53-mutated and rapamycin-resistant WiDr colon adenocarcinoma cells and subcutaneously growing tumors. The spatiotemporal p-mTOR expression *in vivo* was also investigated after PDT. The ability of the mTOR inhibitor rapamycin to enhance the cytotoxic effect of PDT was in addition evaluated *in vitro*.

Experimental Section

Cell Line and Culture Conditions. The colon adenocarcinoma cell line WiDr (ATCC CCL-218), a derivative of HT-29, with a p53 mutation (G → A, Arg273 → His), was used.[20] The WiDr cells were subcultured twice a week in RPMI 1640 medium (Sigma, St. Louis, MO), supplemented with 10% fetal calf serum (GIBCO BRL, Paisley, U.K.), 100 U/mL penicillin and 100 μg/mL streptomycin and 2 mM glutamine (Sigma). The cells were grown and incubated in 75 cm^2 flasks (Nalge Nunc Int., Naperville, IL) at 37 °C in a humidified atmosphere containing 5% CO_2.

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Drugs and Chemicals. $AlPcS_{2a}$ was purchased from Porphyrin Products (Logan, UT). $AlPcS_{2a}$ was first dissolved in 0.1 M NaOH and thereafter diluted in PBS, pH 7.5 to a concentration of 1.25 mg/mL and a final concentration of 0.02 M NaOH. The photosensitizer was light protected and stored at −20 °C until use. Rapamycin was purchased from Sigma (St. Louis, MO) and dissolved in 99.9% dimethylsulfoxide (DMSO) (Sigma) to a final concentration of 4.38 mM and stored as aliquots at −20 °C until use.

***In Vitro* Light Source.** The light exposure of cells in culture was performed by using LumiSource (PCI Biotech, Oslo, Norway). The lamp consists of a bank of 4 light tubes (4 × 18 W Philips Fluotone 18W/950), which emits red light (610−720 nm) filtered through a 620 nm long pass filter (Vink Plast, Kolbotn, Norway). The irradiance varies less than 10% across the whole illumination area (765 cm^2) with an output of 1.5 mW/cm^2. The light source is air-cooled during light exposure, which prevents cells from being exposed to hyperthermia and keeps the irradiance stable.

Photochemical Treatments *in Vitro*. Cells were harvested with 500 μg/L trypsin and 200 mg/mL ethylenediaminetetraacetic acid (EDTA) (Sigma) and seeded out at 2000 cells per well in 96-well plates (Nunclon, Nunc, Roskilde, Denmark) for the MTT-assay, 1000 cells/well in 6-well plates (Nunclon) for colony-forming ability experiments, 50 000 cells/well in 6-well plates for the experiments with Coulter counter and 500 000 cells/wells in Falcon 3003 63 cm^2 wells for sample preparation to SDS−PAGE and Western blotting. The cells were allowed to attach to the bottom of the wells for 24 h prior to start of the experiments. Cells were then incubated with 20 μg/mL $AlPcS_{2a}$ for 18 h followed by 2× wash with culture medium and subsequently a 4 h chase period in drug free medium before the cells were exposed to increasing light doses.

Combination of PDT and Rapamycin. Two different *in vitro* treatment strategies were examined: (i) Rapamycin, at different concentrations as indicated in the figures, was incubated in the medium throughout the entire experiment, i.e. coincubated with PS for 18 h, incubated during PS-free chasing period (4 h), incubated during light exposure, and present until cytotoxic assessments 96 h after light exposure. Total rapamycin incubation time was therefore 5 days. (ii) Alternatively, rapamycin was incubated only directly after light exposure and was present in the medium until cytotoxic evaluation, which gave a total rapamycin incubation time of 4 days.

Statistic Evaluations of the Combined Effect of PDT and Rapamycin. To assess possible synergistic/antagonistic effects when rapamycin was used in combination with PDT we used the parameter $DL = -(\ln S_{comb} - \ln S_{add})$, which is the difference in log values between the observed survival fraction of the combined regimen and the expected survival fraction when rapamycin and PDT are supposed to act independently. The latter term, $\ln S_{add} = \ln S_{PDT} + \ln S_{Rap}$, is given from the single agent experiments at the same doses. The DL values from repeated experiments are averaged for the case of rapamycin either being added after PDT or when it is present both before and after PDT. Significant differences from zero of positive (synergistic) or negative (antagonistic) DL are assessed by two-tailed *t* tests with a significance level of $p = 0.05$.

Cytotoxic Evaluations. Three independent assays were employed to measure the cytotoxic responses of the treatments: (i) The MTT-assay was performed 24 or 96 h after light exposure as indicated in the figure legends. Then the culture medium was removed and new medium containing 0.25 mg/mL MTT reagent was added to the cells. The cells were incubated for 2−4 h and the formazan crystals dissolved in DMSO. Absorbance at 570 nm was used as a measure of cell viability. (ii) Cell counting was performed 96 h after light exposure. The cells were then trypsinized, pelleted, and the pellets dissolved in 20 mL of PBS. Two milliliter portions of the final suspensions were counted by an in-house made Counter coulter. (iii) To verify the MTT assessments, colony forming ability assays were performed 7−10 days post PDT as recently described.[21]

SDS−PAGE, Western Blotting and Immunostaining. Harvesting of *in vitro* growing cells and *in vivo* growing tumors was done at different time points after light exposure, and the relative number of cells in each sample was measured as recently described.[22] Samples were then subjected to SDS−PAGE with 7.5% gels for mTOR- and Akt-analysis and 12% gels for S6-analysis prior to Western blotting. The PVDF transfer membranes (GE Healthcare, Amershamplace, U.K.) were then incubated with different primary antibodies from Cell Signaling Technology (Danvers, MA); mTOR (#2972), p-mTOR (Ser^{2448}) (#2971), Akt (#9272), p-Akt (Ser^{473}) (#4051), p-Akt (Thr^{308}) (#4056) and p-S6 ($Ser^{235/236}$) (#4856). Horse radish peroxidase (HRP) conjugated donkey-antirabbit antibody (Promega, Madison, WI) and horse-antimouse antibody (Cell Signaling Technology) were used as secondary antibodies. ECL plus Western blotting detection systems (GE Healthcare) and Image Quant software (Molecular dynamics, GE Healthcare) were used to detect and quantify the protein bands. Membranes were stained with Ponceau S to verify even loading and transfer of proteins.

Immunohistochemistry (IHC). Five-micrometer sections from formalin-fixed and paraffin-embedded WiDr tumor tissue blocks were cut and mounted on positively charged microscopy slides. The sections were then dried for 45 min at 60 °C, overnight at 37 °C and stored at 4 °C until use. The deparaffinized tissue sections were pretreated in a microwave oven at 750 W for 5 min and 500 W for 15 min in buffer (pH 6; Dako, code no. S1699) for target retrieval before they were incubated with the IHC specific rabbit primary antibody against human p-mTOR (Cell Signaling Technology) (#2976) diluted 1:50 for 30 min at room temperature. The sections were then stained with the Dako-Cytomation EnVision$^+$ System-HRP (DAP) Rabbit (Dako-Cytomation, Inc., Carpentaria, CA) (#K4011), counterstained with hematoxylin, and finally, dehydrated and mounted in Eukitt (Sigma). A negative control was included with the buffer solution only instead of the primary antibody.

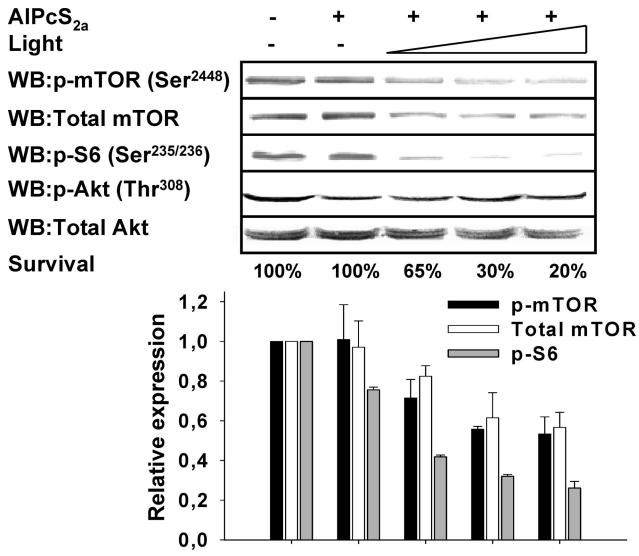

Figure 1. Downregulation of mTOR signaling pathway after PDT. Light dose dependent mTOR signaling 5 min post $AlPcS_{2a}$-PDT. Immunoblots of p-mTOR(Ser^{2448}), total mTOR, p-S6($Ser^{235/236}$), p-Akt(Thr^{308}) and total Akt in cells subjected to increasing light doses with indicated cytotoxicity. The amounts of p-mTOR, total mTOR and p-S6 were quantified as relative to untreated cells and are presented. MTT was assessed at 24 h post PDT. Efficiency of transfer and even loading of lanes were verified by using Ponceau S staining. The data are the average of triplicates based on three independent experiments. Bars, standard errors.

Animal Studies and *in Vivo* PDT. BALB/c (nu/nu) nude female mice were bred at the Animal department of the Norwegian Radium Hospital. The mice were kept under specific pathogen-free conditions, and all procedures involving mice were carried out in agreement with the protocols approved by the Animal Care Committee at the hospital under control by the National Ethical Committee's guidelines on animal welfare. All mice were acclimated in the Animal department for at least 1 week before tumor implantation. The mice were on average 20−25 g (5−8 weeks) at the start of the experiment. WiDr cells from subcutaneous (sc) growing tumors were mixed to homogeneity by a scalpel, and 20 μL of the tumor-tissue suspension was injected s.c. on the left hip of each mouse. Ten mg/kg $AlPcS_{2a}$ was injected intraperitoneally (ip) when the tumors approached a volume of 100 mm^3. The tumor volume was calculated using the following formula:

$$V = (W^2 \times L)/2$$

according to the US National Cancer Institute protocols where W is the width and L the length of the tumors measured 2 or 3 times per week with a caliper. Tumors (75−125 mm^3) were illuminated 48 h after $AlPcS_{2a}$ administration by 670 nm light with an irradiance rate of 100 mW/cm^2 and a total dose of 30 J/cm^2 using a diode laser from CeramOptec Industries Inc. (Bonn, Germany). The animals were protected from light with aluminum foil except above the tumor area including an adjacent rim of approximately 2 mm of normal skin during illumination.

Results

Immediate Response to PDT on mTOR Signaling. The direct response to PDT (by increasing light-doses giving increased cytotoxicity) on p-mTOR and the phosphorylation-status of its downstream effector the ribosomal protein S6 was evaluated *in vitro* 5 min after light exposure (Figure 1). It was demonstrated that after a LD_{35} dose the phosphorylation of mTOR and S6 was attenuated by 30% and 60%, respectively. The reduction in phosphorylated mTOR and S6 increased with increasing PDT doses. Total mTOR was also reduced with increasing light doses. Surprisingly, treatment with the photosensitizer in the absence of light caused a 20% reduction in p-S6, but not in the p-mTOR level (Figure 1).

The WiDr cells were found to continuously express Thr^{308}phoshorylated Akt when incubated in culture medium with 10% FCS. However, no photochemical effects on Thr^{308} phosphorylated Akt nor total Akt were observed after the photochemical treatment (Figure 1) compared to cells that were incubated with photosensitizer only. A Ser^{473} phosphorylation of Akt was detected neither in control nor in PDT-treated cells (data not shown).

Time-Dependent Dephosphorylation of mTOR after Rapamycin or PDT *in Vitro*. Serum starvation of cells overnight before incubation with growth factors is a common method to stimulate phosphorylation of mTOR.[23] However, in WiDr cells activated mTOR could be detected without serum starvation. Four hour incubation of the WiDr cells with 10 nM rapamycin attenuated Ser^{2448} phosphorylation of mTOR by 25% as assessed by Western blot analysis (Figure 2A). A prolongation of the 10 nM rapamycin exposure to 24 h did not further decrease the level of p-mTOR (Figure 2A) nor did increasing rapamycin concentrations up to 100 nM (data not shown). Total mTOR expression was not influenced by the 24 h rapamycin treatment. Twenty-four hours of 10 or 100 nM rapamycin did not induce decreased viability as measured by the MTT method. Sustained incubation of more than 72 h did, however, reduce the viability as presented in Figure 3. The time dependent effects on mTOR were also studied after PDT (LD_{35}) *in vitro*. As can be seen in Figure 2B, downregulation of p-mTOR and total mTOR was transient and returned to

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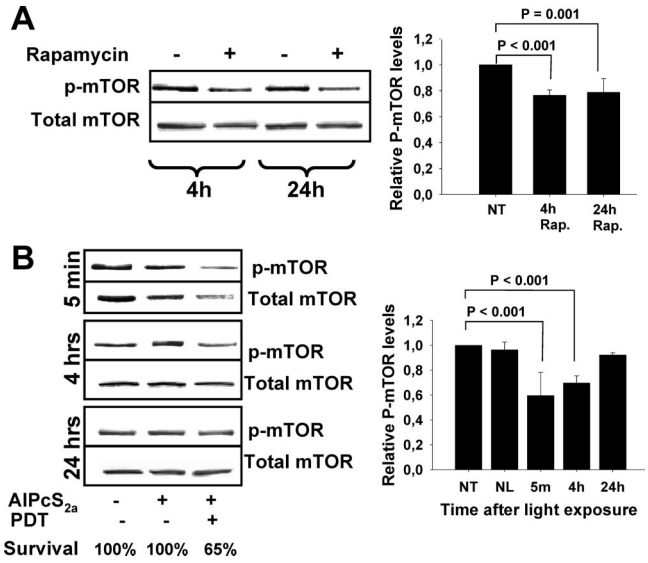

Figure 2. Time-dependent inhibition of mTOR after subtoxic rapamycin or PDT treatment. Total and p-mTOR 4 and 24 h after treatment of WiDr cells with A, 10 nM rapamycin and B, PDT (LD_{35}) 5 min, 4 and 24 h after light exposure. Levels of p-mTOR were quantified as relative to untreated cells. MTT was assessed at 24 h post PDT. Efficiency of transfer and even loading of lanes were verified by using Ponceau S staining. The data are the average values of three independent experiments. Bars, standard errors.

control levels 24 h after PDT. Even loading of proteins was verified by using Ponceau S staining (data not shown).

Rapamycin-Induced Synergistic or Antagonistic Responses in Combination with PDT. The WiDr cells were challenged with a combination of PDT and rapamycin to evaluate possible synergistic effects on cell inactivation. Synergistic effects as measured by the synergy parameter DL (as described in Experimental Section) were observed when the WiDr cells were exposed to rapamycin for 96 h after PDT (Figure 3A and 3B, with corresponding synergy parameters in Figure 3E). The DL values are all positive, which implies synergy with respect to cytotoxicity. This effect is larger for the 4 min light exposure than for the 3 min exposure. The variation with rapamycin concentration is rather small. Except for the DL values at 0.3 nM ($p = 0.10$) and 30 nM ($p = 0.07$) of rapamycin after 3 min of light exposure, all DL values in Figure 3E are significantly positive. Averaging DL over the rapamycin concentrations, the observed cell viability was ~1.5 and 2-fold lower than expected from the calculated additive effects after PDT with 3 and 4 min of light exposure, respectively. In contrast, antagonistic effects were observed in WiDr cells that were exposed to rapamycin throughout the whole procedure, i.e. for 22 h prior to light exposure and until the time of analyses

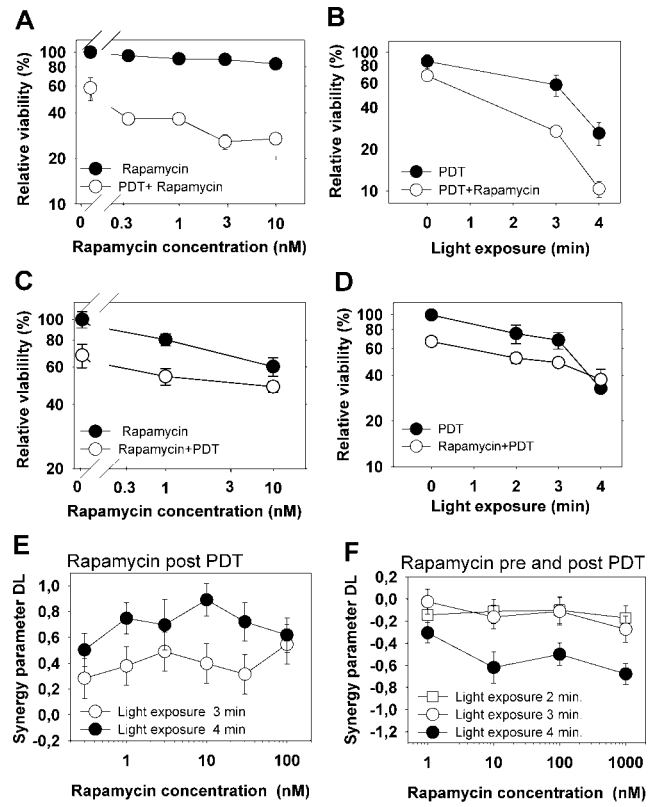

Figure 3. Rapamycin-induced synergistic or antagonistic responses in combination with PDT are dependent on time of rapamycin administration. Cytotoxic effects of PDT alone or in combination with rapamycin after two different experimental treatment regimes. A and B, rapamycin was administrated to the cells immediately after PDT. C and D, rapamycin was present throughout the whole experiment from the initiation of the $AlPcS_{2a}$ incubation until the MTT measurements (96 h post PDT). A and C, light dose was kept constant (LD_{40}) and the rapamycin concentration varied. B and D, the rapamycin concentration was kept constant at 10 nM and the light dose varied. The data are representative results of reproduced experiments and show the average of triplicates presented as compared to untreated control cells. E and F, DL values after combination treatment with constant PDT doses and increasing rapamycin doses. E, experiments where rapamycin was added immediately after light exposure while F, experiments where rapamycin was present both before and after the photochemical treatment. Data are the average of at least three independent experiments. Bars, standard errors.

(Figure 3C and 3D, with corresponding synergy parameters in Figure 3F). Figure 3F shows the DL values obtained from experiments where rapamycin is present both before and after PDT. In contrast to the regime where rapamycin was added after PDT, these DL values are all negative, which implies antagonism, but only treatment with 4 min of light (and the 3 min of light in combination with 1000 nM rapamycin, $p = 0.047$) is significant. Averaging DL over rapamycin concentration gave an antagonistic survival enhancement factor of ~1.15 for 2 and 3 min of light exposure, and ~1.7

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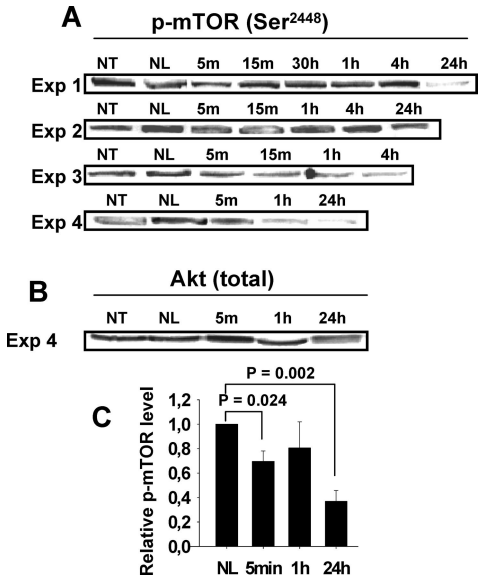

Figure 4. Expression of phosphorylated mTOR *in vivo* after PDT. A, Effects of PDT on the p-mTOR level in subcutaneously growing WiDr tumors. B, Representative blot of total Akt (from experiment 4). Efficiency of transfer and even loading of lanes were verified by using Ponceau S staining. C, Activated mTOR was quantified relative to untreated tumors. Four independent experiments (Exp) were performed. Each protein band represents one tumor. Bars = standard errors.

in the case of the 4 min of light exposure when compared to the expected additive effect.[24] In general, the treatment-dependent observation of synergism or antagonism was highly dependent on the PDT-dose and less dependent on the rapamycin concentration. Rapamycin-induced antagonistic and synergistic responses in combination with PDT were confirmed by counting individual cells using Coulter counter and by colony forming ability measurements as alternative methods for cytotoxicity evaluations.

***In Vivo* PDT-Effects on Downstream p-mTOR.** Mice with subcutaneously growing WiDr tumors were subjected to PDT causing a temporary reduction in tumor volume and a 2−3 week delay in tumor growth as recently described.[22] It was demonstrated that the level of activated mTOR was attenuated by PDT also *in vivo* (Figure 4A and 4C). Five minutes post PDT the p-mTOR level was reduced by 35% compared to nontreated tumors as evaluated on Western blots of whole tumor extracts ($P = 0.024$). At 24 h post PDT the activated mTOR in the WiDr-tumors was further reduced by >75% compared to nontreated control tumors ($P = 0.002$). However, the level of mTOR phosphorylation between 5 min and 24 h post PDT varied strongly among treated tumors as presented in Figure 4A. Figure 4B is a representative blot for total Akt, indicating no change of expression over time post PDT, except a slight decrease after 5 min. However, this was not statistically significant when judging several immunoblots ($P > 0.05$). Corresponding tumor growth data were recently presented.[22]

Spatiotemporal Expression of p-mTOR after *in Vivo* PDT. Analysis of tumor tissue extracts on Western blots as described above provides only information on the presence of phosphorylated mTOR in the whole tissue extracts. To obtain information about the spatiotemporal activation of mTOR at Ser[2448], immunohistochemistry (IHC) on sections from paraffin-embedded WiDr tumor xenografts was performed. In control tumors, phosphorylation of mTOR was found to be heterogeneously distributed all over the tissue (Figure 5A). At 1 and 4 h post PDT, p-mTOR was still detectable throughout the tumor, especially in the viable islands of colon adenocarcinoma cells. Twenty-four and 48 h after PDT almost no staining of p-mTOR was observed in the tumor except in the peripheral area still showing a similar level of p-mTOR expression to that of control tumors (Figure 5A and 5B). The fraction of necrotic tissue, in particular in the central area, was increased from 24 to 48 h post PDT. Additional IHC sections of p-mTOR expression from the xenograft tissues are shown in Supplementary Figures 1−3 in the Supporting Information (controls, 1, 4, 24 and 48 h post PDT).

Interestingly, a significant expression of p-mTOR was observed in the epidermis overlaying the tumors, while the dermis was negative for activated mTOR. In addition, some of the hair follicles were also highly positive for p-mTOR (Figure 5C).

Discussion

The tumor-suppressor p53 inhibits mTOR activity as a part of its action mechanism.[9] p53 is mutated in about 50% of all cancers.[25] Therefore, we found it interesting to study the PDT effect on mTOR in the p53 mutated colon adenocarcinoma cell line WiDr compared to the outcome of rapamycin treatment. Rapamycin is a specific inhibitor of the mTORC1 complex and a cell type dependent inhibitor of Akt and mTORC2.[26]

In the present study we show that 4 and 24 h rapamycin exposure reduced the phosphorylation of mTOR in the WiDr cell line by 25%. The rapamycin treatment of WiDr cells induced low cytotoxicity, i.e. <30% of the cells were inactivated after 96 h treatment with 10 nM rapamycin, which is in accordance with a recent report on rapamycin treatment

(24) If the additive cytotoxic effect of PDT and rapamycin were caused by the same mechanism (named Mode II by Steel and Peckham) instead of by mutually independent mechanisms (Mode I), the expected survival fraction would be larger and correspond to a larger synergy effect in the case when rapamycin is used after PDT. Conversely, the additive survival fraction would be smaller in the Mode II calculations and indicate a larger antagonism when rapamycin is present during the whole experiment compared to the Mode I calculation. Steel, G. G.; Peckham, M. J. Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity. *Int. J. Radiat. Oncol. Biol. Phys.* **1979**, *5*, 85–91.

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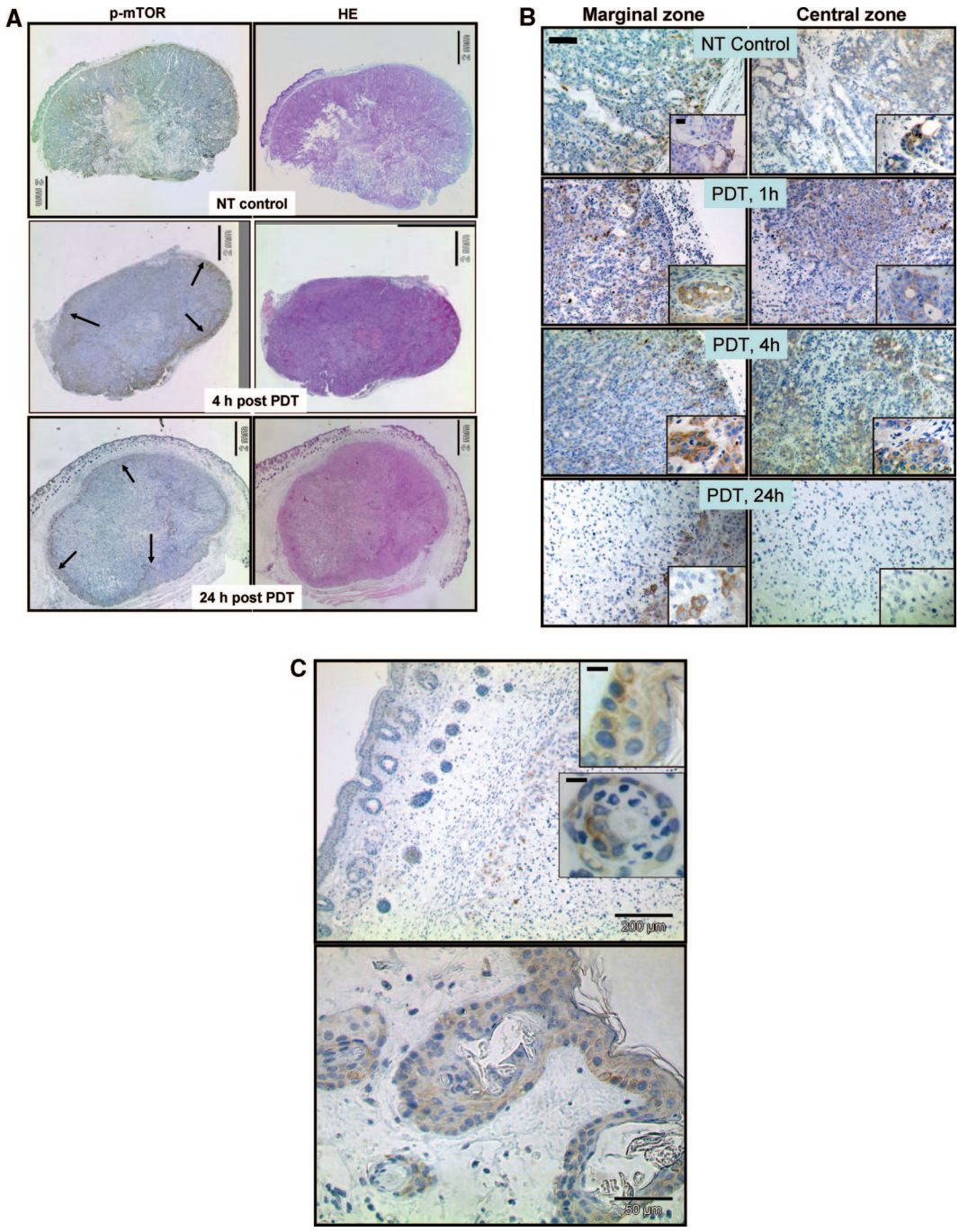

Figure 5. Spatiotemporal expression of mTOR post *in vivo* PDT. Immunohistochemistry of p-mTOR (Ser2448) in WiDr tumors post PDT. A, Nontreated (NT) and PDT-treated animals 4, and 24 h post light exposure. Arrows indicate the rim of p-mTOR post PDT. Bars = 2 mm. B, Cellular and subcellular localization of p-mTOR in nontreated (NT) and PDT- treated tumors 1, 4 and 24 h post light exposure. Left panel is from the marginal zone and right panel is from the central zone of tumor. Small panels (magnifications) are from the corresponding areas. Bar in upper left panel (large window) = 100 μm. Small panel bar = 20 μm. C, p-mTOR staining of skin above tumor. Large panel bar = 2000 μm and small panel bar = 10 μm. The upper small panel is a magnification of epidermis, while the other small panel shows a hair follicle. Control IHC, see Supplementary Figure 3C in the Supporting Information.

of colon carcinomas, including the WiDr cell line.[27] PDT effects on mTOR are here demonstrated for the first time and shown to induce rapid inhibition of Ser^{2448} phosphorylated mTOR both *in vitro* and *in vivo*. In addition, PDT induced dephosphorylation of S6, an effector protein downstream of S6 kinase that is downstream of mTOR. Akt is located both upstream and downstream of mTOR.[28,29] Interestingly, Akt was not affected by $AlPcS_{2a}$-PDT, sug-

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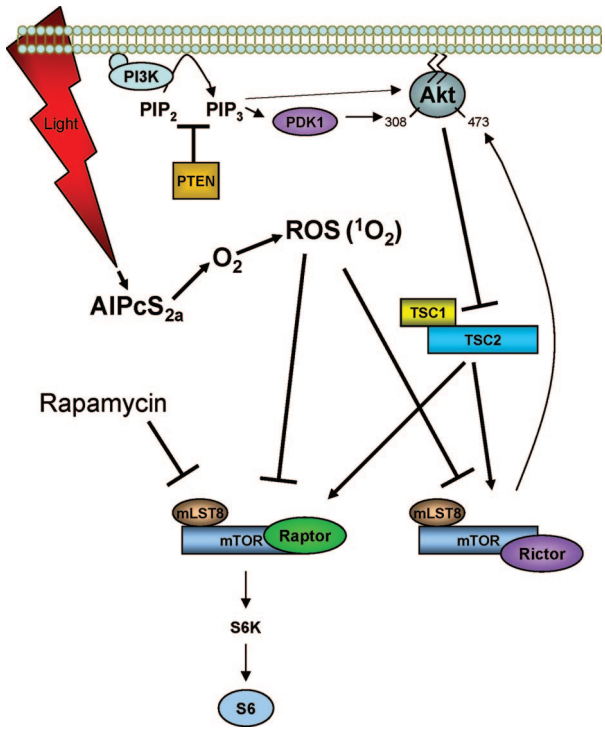

Figure 6. Schematic model of the mTOR signaling pathway and its inhibition by rapamycin and the suggested role of $AlPcS_{2a}$-PDT. Upon light activation, lysosomal/ER-associated $AlPcS_{2a}$ mediates photochemical generation of ROS, of which singlet oxygen ($^1O_2$) is the most abundant, and thereby cause a direct oxidative damage to mTOR. This attenuates the downstream signaling of mTOR, including deactivation of S6.

gesting that $AlPcS_{2a}$ is not colocalized with Akt. The present results show that the reduction in p-mTOR after PDT was larger than that observed after rapamycin treatment in the WiDr cells. The lack of additional attenuation of mTOR phosphorylation by increasing the rapamycin treatment concentration above 10 nM indicate that mTORC1 activity is completely inhibited by rapamycin and that the remaining p-mTOR is due to phosphorylated mTOR in the mTORC2 complexes. The differences in effects on p-mTOR between rapamycin and PDT reported here may therefore be caused by PDT-mediated attenuation of both mTORC1 and mTORC2 compared to rapamycin which mainly acts on mTORC1. However this has to be further explored and confirmed by immunoprecipitation of mTORC1 and mTORC2 and further evaluation of PDT mediated attenuation of mTOR in both complexes.

The present results show a reduction in total mTOR and mTOR signaling immediately after PDT *in vitro*, which may indicate that the photochemical treatment causes a direct oxidation of mTOR. PDT induces ROS of which singlet oxygen ($^1O_2$) is suggested to dominate and cause oxidation of surrounding biomolecules. Singlet oxygen has an extremely short lifetime (<10−40 ns) and diffusion length (<10−20 nanometers) in cells. Hence, the cytotoxic action of PDT takes place in the very close vicinity of the subcellular site of $^1O_2$ formation.[30−32] The photosensitizer used in this study, $AlPcS_{2a}$, is shown both *in vitro* and *in vivo* to localize to endosomes and lysosomes.[33−35] In addition, TOR2 has been shown to be localized in lysosomes/vacuoles in yeast.[36] Based on these observations, we suggest that mTOR and $AlPcS_{2a}$ may be colocalized in endocytic vesicles of cells not exposed to light. Furthermore, it has been documented, both *in vitro* and *in vivo*, that $AlPcS_{2a}$ is released from the endocytic vesicles during light exposure, and relocalizes to other cellular compartments except the nucleus.[34,35] In mammalian cells, mTOR has been suggested to be associated with intracellular membranes of which endoplasmatic reticulum (ER) has in particular been documented or to the plasma membrane and a minor fraction in the cytosol.[37,38] The photosensitizer $TPPS_{2a}$, which has the same cellular localization pattern as $AlPcS_{2a}$, relocalizes to ER upon light exposure and it has been suggested that PDT-induced targeting of ER is of importance for the cytotoxic

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effect of $TPPS_{2a}$-PDT.[39] mTOR is shown to be localized to the cytosolic side of the ER and the Golgi apparatus in cells.[40,41] Therefore, based on the data in the literature and our observations, we suggest that mTOR is directly targeted by PDT using photosensitizers that are localized to ER and endolysosomal compartments (Figure 6).

Our results indicate that in the presence of serum neither PDT nor rapamycin as a single treatment manages to completely abolish mTOR phosphorylation in WiDr cells. We therefore hypothesized that PDT−rapamycin combination therapy could be a strategy to enhance the cytotoxic effects in a synergistic manner. Synergistic effects were indeed observed when rapamycin was administered after the photochemical treatment. As shown in Figure 2B, the PDT induced attenuation of p-mTOR was transient and the level of activation was restored to the level in untreated cells 24 h after light exposure. Administration of rapamycin after PDT probably induces a more sustained attenuation of mTOR, which may explain the observed increase in cytotoxicity after the PDT−rapamycin combination treatment. In contrast, the combination treatment of rapamycin and PDT resulted in antagonistic effects when rapamycin was present throughout the whole PDT procedure including prelight treatment. As yet, we do not know the mechanism behind the antagonistic effect of rapamycin. One reason could be that rapamycin bound to mTOR may block PDT-induced attenuation of the protein. Another possible mechanism of antagonism could be that rapamycin cause reduced binding and uptake of the photosensitizer. Cells in the G1 phase of the cell cycle are less sensitive to PDT compared to cells in other phases.[42] As rapamycin inhibits the traverse from G1 to S phase and therefore causes accumulation of the cells in G1, this may also explain the antagonistic effects observed when rapamycin is delivered to the cells prior to PDT.

IHC analysis of the tumor xenografts collected at different time points post PDT revealed a spatiotemporal dependent level of p-mTOR expression. One and 4 h post PDT the expression of p-mTOR was heterogeneous throughout the tumor tissue. However, 24 and 48 h post PDT most of the p-mTOR expression in the WiDr tumors disappeared, including areas with high density of viable cells in the central part of the tumor, consistent with the finding from the Western blots. However, in a thin continuous circumferential lining of cells in the rim of the tumors a significant fraction of cells expressed p-mTOR. Although tumors were not collected later than 48 h post PDT, the size of the necrotic areas was macroscopically increased at 98 h after PDT in different types of tumors (data not shown). The relative high level of p-mTOR in the margin of the tumor after PDT was surprising. The distal part of the tumor, closest to the skin, is the area of the tumor exposed to the highest fluence of light, and thereby the highest PDT doses assuming that the photosensitizer is evenly distributed in the tumor. One should therefore expect that the distal part of the tumor should express the lowest level of p-mTOR. Interestingly, the mTOR activity in the rim of the tumor seems to be independent of the PDT-dose received since there is apparently a similar expression of p-mTOR throughout the periphery of the tumor. Based on the current data, we cannot explain the rationale of the observed p-mTOR rim effect. Vascular shutdown is assumed to be one of the antitumor mechanisms of PDT, and it has been reported by others using vascular-targeting agents that a rim of viable cells is left after treatment.[43]

In conclusion, $AlPcS_{2a}$-PDT inhibits activation of both mTOR signaling in the rapamycin resistant and p53-mutated colon adenocarcinoma cell line WiDr and in a WiDr xenograft model in mice, while neither the expression of total Akt nor Akt-activation is affected. Treatment of the WiDr cells with rapamycin after PDT led to synergistic cytotoxicity, while pretreatment with rapamycin prior to PDT gave antagonistic effects *in vitro*. We propose that PDT is an alternative mTOR targeting approach and should be further explored in combination with rapamycin or its analogues in preclinical settings, in rapamycin resistant or sensitive model systems. The present results suggest attenuation of mTOR signaling as an important mechanism for PDT mediated cell death. The implication of mTOR in metastasis and angiogenesis warrants evaluation of PDT as a means to strengthen the anticancer effects of rapamycin analogues.

Abbreviations Used

$AlPcS_{2a}$, disulfonated aluminum phthalocyanine with the sulfonate groups on adjacent phthalate rings; DL, difference in log values; LD_x, light dose killing x% of the cells; mTOR, mammalian target of rapamycin; mTORC1/2, mTOR complex1/2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDT, photodynamic therapy; ROS, reactive oxygen species; $TPPS_{2a}$, meso-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings.

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Supporting Information Available: Figure SI 1: IHC of p-mTOR. WiDr tumor tissues from nontreated (NT) and PDT-treated animals 1, 4, 24 and 48 h post light exposure. P-mTOR is shown in left panels and corresponding HE staining on the right panels. Figure SI 2: IHC of p-mTOR. Reproduction of nontreated and treated (24 h post PDT) WiDr tumors. Arrows indicate the rim of p-mTOR post PDT. Figure SI 3: Control IHC (24 h post PDT) with an irrelevant primary antibody not targeting phosphorylated mTOR and the same secondary antibody as used in Figure 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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