Effect of a photoactivated rhodium complex in melanoma

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***cis*-Dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride (OCTBP) is an octahedral complex that has been shown to react with nucleic acids when irradiated with light. Earlier studies on its phototoxicity toward human esophageal, bladder, pancreas, and colon cancer cells have been extended to in-vitro and in-vivo evaluation of its effect on malignant melanoma cells using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium proliferation assay and xenograft model of melanoma. Tumor volume and the percentage of tumor growth delay were used to assess the antitumor effect of light-activated OCTBP. Terminal deoxynucleotidyl transferase dUTP nick-end labeling assays and immunohistochemistry were also performed on tumor tissue samples. Photoactivated OCTBP was found to inhibit melanoma cell growth by 40% at 100 μmol/l concentration. Phototherapy with OCTBP was most efficacious with optimal percentage treated/control values observed early in the course of the experiment. At the end of the experiment (60 days), there was a 50% tumor inhibition compared with vehicle or drug controls. OCTBP treatment resulted in a tumor growth delay of 16 days (tumor growth of 24%) compared with the untreated control, whereas cisplatin resulted in 10 days tumor growth delay. This antitumor activity was found to be closely associated with the induction of apoptosis. The use of photoactivated OCTBP as an adjuvant therapy for the treatment of melanoma may provide a new targeted therapy to prevent progression of this disease. *Anti-Cancer Drugs* 22:896–904 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The photochemotherapeutic process known as photodynamic therapy (PDT) is an emerging form of therapy that may be used for the treatment of skin cancers. In this form of therapy, a drug, termed a photosensitizer, is administered to a patient, and upon the drug entry and accumulation into the cells, it is activated by light to start cellular processes that lead to tumor cell death [1]. The need for light activation of the photosensitizer, after its accumulation in the tumor cells, provides this approach with many advantages over other modes of cancer therapy. The most important are the lack of systemic toxicity (as is the case with chemotherapy), selective targeting, the ability to instantaneously 'turnoff' activity, and the ability to re-treat with minimal invasiveness. PDT has been approved by the Food and Drug Administration in the USA for the treatment of many diseases, including actinic ketosis, esophageal and lung cancers, and age-related macular degeneration [2].

In PDT, the phototherapeutic drug acts as a 'photosensitizer', that is, it interacts with oxygen, when irradiated, to form cytotoxic reactive oxygen species such as singlet molecular oxygen, hydroxyl radical, and/or superoxide anions. These cause the destruction of tumor cells by apoptosis or necrosis and damage to the tumor microenvironment due to oxidative stress [3]. Although PDT has shown great clinical promise in the treatment of esophageal cancer [4], early lung cancer [5], skin cancers [6], bladder cancer [7], and head and neck cancers, tumor resistance, due to a lack of oxygen (hypoxia), and the indiscriminate damage to the surrounding tissue from reactive oxygen species, are problematic. Hypoxia and oxidative stress result in many molecular and physiological processes that may lead to neovascularization and angiogenesis [8]. Neovascularization supplies the tumor cells with required nutrients and oxygen, thus allowing these cells to grow and invade nearby tissues.

Recently, several novel organometallic photoactive compounds have been synthesized in our laboratory [9,10], which do not rely on the generation of reactive oxygen species to effect cell death. Instead, these chemicals bind with, and nick, DNA and RNA after light activation, making them candidate phototherapeutic anticancer drugs. Although the parent complex *cis*-dichlorobis (1,10-phenanthroline) rhodium(III) chloride showed minimal dark association with DNA, due to the absence of a ligand capable of intercalation, and lacked the hydrophobicity needed for the uptake by mammalian cells, two second-generation compounds showed appreciable in-vitro efficacy against DNA viruses and against several tumor cell lines when activated by 311 nm [ultraviolet (UVB)] light [9,10]. One in particular,

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cis-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride (OCTBP), is a methylated analog of *cis*-dichlorobis (1,10-phenanthroline) rhodium(III) chloride, which is substantially more photoreactive than the parent complex, is sufficiently hydrophobic to be taken up by tumor cells, and provides a higher level of ground-state association with double-stranded DNA. OCTBP forms covalent bonds to deoxyguanosine when irradiated with the nucleoside. Irradiation of OCTBP in the presence of the Kb or M109 tumor cell lines, using narrow-band UVB (311 nm) irradiation, initiates a considerable amount of phototoxicity [10].

Although PDT is used for the treatment of many cancers [4–7], and is approved by the Food and Drug Administration to be used in many other skin diseases [2], not much research has been conducted to examine its effect in malignant melanoma. Malignant melanoma constitutes only 10% of skin malignancies, but results in more than 80% of all skin cancer deaths [11]. These deaths are due to the fact that melanoma is highly resistant to radiation and chemotherapy treatments. Although primary malignant melanoma can be treated by surgery alone, advanced malignant melanoma is difficult to treat and surgery does not increase patient survival [12]. Therefore, this study was designed to evaluate the OCTBP-mediated phototoxic effect on various solid tumor cells, *in vitro* and *in vivo*, and to elucidate the mechanism by which OCTBP achieves in-vivo phototoxicity.

Materials and methods

Formulation of *cis*-dichlorobis (3,4,7,8-tetramethyl-1, 10-phenanthroline) rhodium(III) chloride

OCTBP was prepared as previously described [10]. The lyophilized powder of OCTBP was reconstituted with sterile dimethylsulfoxide (Sigma-Aldrich, St. Louis, Missouri, USA) at a concentration of 50 mmol/l and diluted either in Roswell Park Memorial Institute medium for the in-vitro proliferation assays, or in 5% dextrose (Hospira, Inc., Lake Forest Illinois, USA) for in-vivo animal studies.

Photochemistry

Photolyses used monochromatic low-pressure mercury lamp irradiation at 311 nm. As previously described, all experiments used a turntable arranged such that multiple samples could be irradiated in an identical manner [10]. For the murine experiments, the mouse was placed on the turntable covered by a protective cloth with an opening of 0.78 cm^2 in size so that only the implanted tumor was exposed to the irradiation, thus mimicking the actual phototherapy treatment.

Cell cultures

Nasopharyngeal carcinoma (Kb), pancreatic cancer (Paca-2 and PANC-1), bladder cancer (UMUC3 and HT1376), colon cancer (HT29 and HCT116), and melanoma (SKMEL-5 and UACC62) cells were newly obtained from the American Type Culture Collection (Rockville, Maryland, USA), except Kb cells that were from an old batch. The Paca-2 and PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, Missouri, USA) with 10% fetal bovine serum (Invitrogen, Carlsbad, California, USA) and L-glutamine (2 mmol/l). HT1376, UMUC-3, and SKMel-5 cells were cultured in minimum essential medium (Sigma) supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/l), and sodium pyruvate (1 mmol/l; Sigma). Colon cancer cells, HT29 and HCT116, were grown in McCoy's 5A medium (Sigma, St. Louis, Missouri, USA) with 10% fetal bovine serum and L-glutamine (1.5 mmol/l). UACC62 cells were cultured in the Roswell Park Memorial Institute medium-1640 with 10% fetal bovine serum and L-glutamine (2 mmol/l). All cells were maintained in a humidified incubator containing 5% CO_2 at 37°C.

Animals

All animal experiments were performed at the Purdue Cancer Center for Research Drug Discovery Shared Resource with the approval of the Purdue Animal Care and Use Committee. Experiments were conducted first in athymic mice and later switched to severe-combined immunodeficiency (SCID) mice. Male athymic mice (nu/nu, 5–6 week old) or SCID mice were obtained from the National Cancer Institute (Bethesda, Maryland, USA). Mice were housed under pathogen-free conditions in microisolator cages with laboratory chow and water ad libitum. The weight of the mice at the time of treatment ranged from 20 to 23 g.

In-vitro studies

The in-vitro cytotoxic effects of OCTBP were evaluated on bladder, pancreatic, colon, and melanoma cells following the protocol described previously [10]. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay. In brief, cells were plated into 35×10 mm tissue culture dishes at a density of 5×10^4 cells per dish for the HT29, Paca-2, HT1376, and PANC-1 cells, and at a density of 3×10^4 cells per dish for the HCT116, UMUC3, UACC6-2, and SKMEL-5 cells. All dishes were incubated for 24 h at 37°C with 5% CO_2 and 90% humidity before a photolysis experiment. The medium was removed from each dish after 24 h and the dishes were washed with 1 ml of phosphate-buffered saline (PBS). OCTBP (2 ml) in media was added and the dishes were incubated for 3 h at 37°C. The same concentration of diluted DSMO in media without OCTBP served as a vehicle control. After 3 h of incubation with or without the drug (vehicle control), the medium was removed and the dishes were washed with 1 ml of PBS. After washing, 1 ml of PBS was added to each dish. The dishes were then placed in the photolysis chamber and were irradiated at 311 nm for 30 min. After this, the PBS was removed and fresh medium was added. The dishes were

then incubated again for 72 h. The cell numbers were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay according to the manufacturer's instructions. The color intensity was measured at 570 nm on a molecular devices kinetic microplate reader (Molecular Device, Sunny, California, USA). Each experiment was repeated three times to confirm reproducibility.

Maximum tolerated light dose

These experiments were conducted to determine the maximum light exposure the mice could tolerate without developing erythema or burn from the light. Mice were anesthetized with ketamine/xyalizine given by intraperitoneal injection. Mice were then placed in the photolysis chamber and the exposed skin was irradiated for increasing periods of time. Twenty-four hours after irradiation, the irradiated skin was examined and scored subjectively according to the following scale: 0 = no erythema; 1 = just perceptible erythema (minimal erythema dose); 2 = mild erythema; 3 = marked erythema; 4 = marked erythema and slight edema; 5 = marked erythema and strong edema; and 6 = bullous reaction.

Maximum tolerated drug dose

The maximum tolerated dose (MTD) of OCTBP was determined for two routes of administration. Mice were injected intravenously (i.v.) or intraperitoneally (i.p.) with either single dose or multiple doses of OCTBP daily. Doses ranged from 0 to 30 mg/kg for i.p. injections or 0–6 mg/kg for i.v. injections. For cisplatin, the MTD was determined in a previous study in our laboratory. Mice were monitored for visible signs of toxicity and were weighed daily. The MTD was defined as the highest dose at which there was no death or less than or equal to 20% body weight loss from the pretreatment animal weight.

Optimal time delay between administration of the drug and irradiation

To determine the optimal time needed after drug administration before irradiation to achieve maximum tumor inhibition, mice were injected subcutaneously with approximately 5×10^6 SKMEL-5 melanoma cells. When the tumor volumes were 50–100 mm^3, as determined by caliper measurements, the mice were randomly assigned to one of the treatment groups ($n = 5$ each). Mice that failed to grow tumors, or had a tumor of more than 100 mm^3, were not included in the randomization (such mice totaled up to 20% of those inoculated). Mice were kept in the dark or irradiated at 30 min, 1, and 2 h after i.p. injection of OCTBP solution, and another group was kept in the dark or irradiated at 10, 30 min, or 1 h after OCTBP i.v. injection. The drug doses were identical among treatment groups at the MTD values. After treatment, the mice were allowed to recover from anesthesia and their tumor volumes were measured twice weekly, and tumor volumes (mm^3) were calculated according to the formula: $V = L \times W^2/2$, where L is length (longest dimension) and W is width (shortest dimension). Mice body weights were recorded once weekly. This procedure was followed weekly until the tumor in mice reached 1000 mm^3, at which point they were killed by CO_2 asphyxiation.

In-vivo phototherapeutic efficacy of *cis*-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride

This study was designed to give the percentage of tumor inhibition and tumor growth delay, which was used as the criteria to determine the antitumor efficacy of OCTBP. The in-vivo efficacy of OCTBP was determined using only the SKEMEL-5 melanoma cells. Melanoma xenografts were induced as above and treatment with OCTBP was initiated similarly when tumors reached a volume of 50–100 mm^3. The mice were then randomized and 10 mice were placed per group, defined as follows: group 1, no light or drug and injected only with the vehicle solution (vehicle control); group 2, treated with light only (light control); group 3, treated with OCTBP, but no light (drug control); group 4, treated with OCTBP and light (experimental group); and group 5, treated with cisplatin (no light; chemotherapy group). Mice with outlier tumor sizes (larger or smaller) were not included in the study. Treatment for these experiments was initiated using doses at the maximum or below MTD as determined previously. Mice were anesthetized and placed into the photolysis chamber. The exposed tumor mass was irradiated for 30 min after drug treatment as determined in previous studies. The mice were observed daily and were weighed once per week. Tumors were measured twice a week. The treatment with OCTBP was repeated five times during the course of the experiment. Cisplatin was given in three doses, 1 week apart. Individual mice were killed when their tumors reached a volume of 1000 mm^3. Mice whose tumors did not reach 1000 mm^3 by 60 days after cell inoculation were killed at that time (60 days). Each mouse underwent complete necropsy with tumor tissue collection. Tumor tissues were then immediately immersed in 10% neutral-buffered formalin (for histopathology and immunohistochemistry evaluations).

Immunohistochemistry

Immunohistochemistry was performed as described previously [11]. Formalin-fixed, paraffin-embedded tumor tissue sections (5 μmol/l) were mounted on charged SuperFrost slides. Sections were then deparaffinized and rehydrated and heat-induced epitope retrieval was performed in citrate buffer, pH 6 (S2369; Dako Corporation, Carpinteria, California, USA), using a food steamer for 20 min at 90–95°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, followed by the application of a nonspecific protein-binding block (X0909; Dako Corporation). Sections were then incubated in primary antibody Ki67 monoclonal mouse anti-human

proliferating nuclear protein clone 7B11 (Zymed Laboratories, Carlsbad, California, USA) overnight at 4°C. Simultaneously, serial sections were used as a negative control and were incubated with normal mouse serum. The antibodies were diluted 1 : 100 in PBS. The immunoreactivity was detected using the labeled streptavidin–biotin–peroxidase method followed by visualization with 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, California, USA). Tissues were counterstained with hematoxylin (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Two investigators reviewed all slides independently. The estimated percentage of cells labeled and the labeling intensity of positive cells for Ki67 were recorded. Intensity was graded on a scale of 0–3, with 0, no staining; 1, equivocal staining; 2, moderate-to-intense staining, and 3, high-intensity staining. Semiquantitative immunohistochemistry scores were calculated by multiplying the estimated percentage of cells labeled by the intensity of labeling.

Effects of *cis*-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride in inducing apoptosis in SKMEL-5 xenografts

Apoptosis was detected in xenograft tissues by a terminal deoxynucleotidyl transferase-mediated nick-end labeling assay using the Apoptag Peroxidase in-situ Apoptosis Detection kit (Intergen Co., Purchase, New York, USA) as previously described by Mohammed *et al.* [13]. The number of positive-stained tumor cells was recorded in a minimum of five fields at $\times 400$, and the apoptotic index (percentage of apoptotic tumor cells) was determined. Two observers examined each section and the results were averaged. Induction of apoptosis was defined as more than or equal to two-fold increase in apoptotic index.

Data analysis

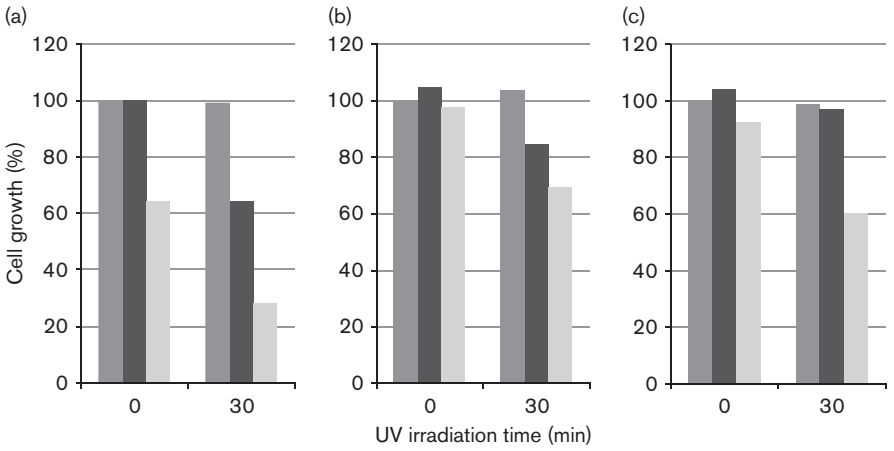
For in-vitro cytotoxicity experiments, bar graph curves were generated from the mean of triplicate determinations and the level of cell growth inhibition was obtained using Microsoft Office Excel. For efficacy studies, tumor growth delay (days) was calculated using the following formula: T – C, where T is the median time in days for the treated tumor to reach 500 mm^3 and C is the median time in days for the untreated tumor to reach 500 mm^3. Tumor growth delay was also expressed as the percentage by using the following formula: $[(T-C)/C] \times 100$.

Results

In-vitro studies

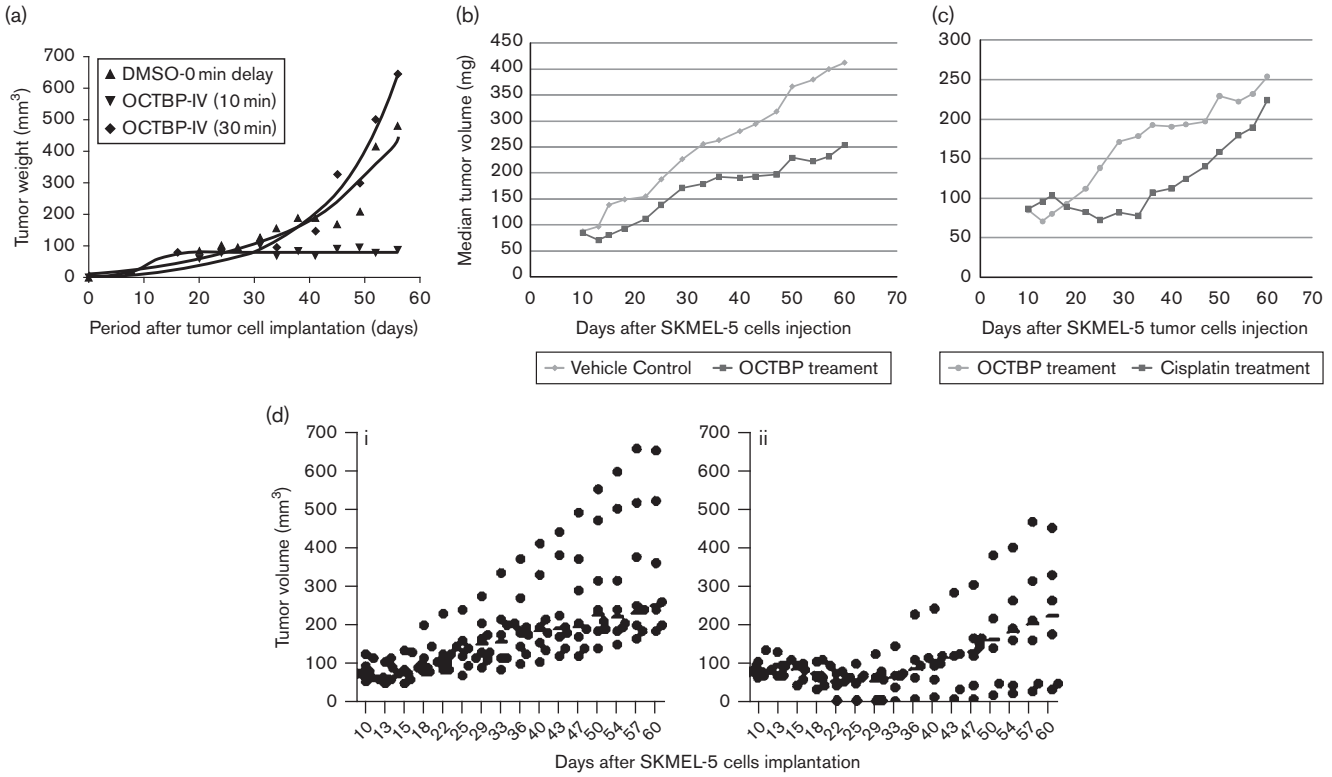
Initially, we confirmed our previous results by examining the phototoxic effect of OCTBP on Kb cells, and then on Paca-2, PANC-1, UMUC, HT1376, HT29, HCT116, SKMEL-5, and UACC62 cells. Kb cells were highly susceptible to the phototoxic effect of OCTBP, with approximately 35 and 75% cell death compared with controls for both concentrations examined (50 and 100 μmol/l; Fig. 1a). However, at 100-μmol/l concentration there was approximately 40% cell death due to the drug in the absence of light (dark toxicity), similar to results obtained in our previous studies. The HCT116 and PANC-1 cells showed no noticeable growth inhibition and the UMUC3, HT1376, HT29, and UACC62 cells showed growth inhibition, but again with some dark toxicity (data not shown). The SKMEL-5 and Paca-2 cells

Fig. 1



Effect of photoactivated *cis*-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride (OCTBP) on Kb nasopharyngeal (a), Paca-2 pancreatic (b), and melanoma (SKMEL-5; c) cell growth. Cells were treated with OCTBP for 3 h. After removal of OCTBP, cells were either not irradiated (0 time), or irradiated at 311 nm for 30 min (30 min time) and allowed to incubate for 72 h after adding fresh media. (a) Kb cells were highly susceptible to the phototoxic effect of OCTBP with approximately 35 and 75% cell death at both drug concentrations (50 and 100 μmol/l) tested; however, there was 40% death due to dark toxicity at 100 μmol/l. (c) Photoactivated OCTBP had minimal inhibitory effect at 50 μmol/l and marked inhibitory effects at 100 μmol/l without much dark toxicity in SKMEL-5 cells.

Fig. 2



(a) Tumor volumes of subcutaneous SKMEL-5 xenograft after i.v. injection of *cis*-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride (OCTBP) at its maximum tolerated dose (4 mg/kg of body weight) and irradiation of the tumor at 311-nm light at 0, 10, and 30 min after the drug administration. A 10-min wait time after drug administration achieved the greatest tumor inhibitory effects compared with no wait control or a 30-min wait time. (b) Tumor volumes of SKMEL-5 xenograft treated with photoactivated OCTBP. Cells were implanted at 5×10^5 cells per 0.1 ml subcutaneously in severe-combined immunodeficiency mice. When the tumor volumes reached 50–100 mm^3, mice were randomized into four groups and treated as follows: no drug, no light vehicle control; no drug, only light control, only drug, no light control; and drug and light experimental group. Drug concentration used was 4 mg/kg of body weight and tumors were irradiated at 311-nm light after a 10-min wait time. The treatments were initiated at day 11 and repeated at day 14, 20, 26, and 35. (c) Tumor volumes of SKMEL-5 xenografts after treatment with photoactivated OCTBP and cisplatin. The treatment with photoactivated OCTBP was repeated five times during the experiment. Cisplatin was given in three doses (6 mg/kg of body weight), 1 week apart. At the end of the experiment, no significant differences in tumor volume were seen between tumors treated with photoactivated OCTBP (250 mm^3) or cisplatin (225 mm^3). (d) Tumor volumes of each SKMEL-5 xenograft after treatment with photoactivated OCTBP (10 mice; D-1) and cisplatin (eight mice; D-2). Five mice in the photoactivated OCTBP treatment group were clustered together with similar tumor volumes (100–200 mm^3); three mice had larger tumors (300–700 mm^3), and two mice died during experimental manipulation. In cisplatin-treated group, two mice were cured (no tumor), two had tumor volumes of 150–250 mm^3, two had larger tumor volumes of 300–450 mm^3, and two mice died due to drug toxicity.

were the most responsive to the phototoxic effect of OCTBP at 100-µmol/l concentration. Figure 1b shows the phototoxic effect of OCTBP on SKMEL-5 cells. OCTBP at 100 µmol/l resulted in 40% cell death in the presence of light, with minimal dark toxicity.

Maximum tolerated light dose

Mice were irradiated at 311 nm for 0, 5, 10, 20, 30, and 45 min. No skin reaction was observed at all points examined.

Maximum tolerated drug dose

To determine the MTD of OCTBP without the light effect, nude and SCID mice were injected with OCTBP solution dissolved in DSMO and diluted in 5% dextrose, either intravenously at doses of 0, 1, 2, 4, or 6 mg/kg of body weight, or i.p. at doses of 0, 5, 10, 20, and 30 mg/kg of body weight. An intravenous dose of 4 mg/kg and an intraperitoneal dose of 20 mg/kg were determined to be the MTD doses, whether these were given in a single dose or as multiple doses. These doses resulted in no toxicity, weight loss, or change in animal behavior and resulted in no animal death; therefore, these were chosen for subsequent studies. The MTD for cisplatin has been determined in athymic mice in a previous study (unpublished data) and was found to be 6 mg/kg of body weight, administered three times, 1 week apart.

Optimal time delay between administration of the drug and irradiation

An optimal time delay was determined using tumor-bearing mice treated at the MTD of OCTBP. Time points were for

i.v., 0, 10, and 30 min, and for i.p. 0, 30 min, 1, and 2 h, after drug treatment. Figure 2a shows the response of tumors treated with OCTBP and exposed to light at 0, 10, and 30 min after i.v. administration of the drug. The combination of a drug dose of 4 mg/kg administered intravenously, with a 10-min delay before light exposure, or drug (20 mg/kg) administered i.p. with a 2-h delay (data not shown), were found to significantly reduce tumor volume compared with no wait (0 time) or 30-min wait time.

In-vivo *cis*-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride phototherapeutic efficacy

The phototherapeutic efficacy of OCTBP was determined at its MTD dose value (4 mg/kg) by i.v. injection, using multiple dosing in a malignant melanoma SKMEL-5 xenograft. As shown in Fig. 2b, OCTBP inhibited tumor growth as evidenced by the volume of SKMEL-5 xenografts in SCID mice. OCTBP was injected i.v. at 11, 14, 20, 26, and 35 days and subsequently photoactivated at each time point. After the first dose, the median tumor volume of photoactivated OCTBP-treated mice decreased by 30% compared with light and vehicle control groups. Curiously, there was some decrease in tumor volumes in mice treated with the drug without light treatment (data not shown). At the end of the experiment (60 days), there was almost 50% tumor inhibition compared with both vehicle and drug-only controls.

Figure 2c shows tumor volumes of the photoactivated OCTBP-treated group compared with a cisplatin-treated group. Cisplatin was administered i.p. at 13, 21, and 28 days after tumor implantation. In comparison with OCTBP, by the end of 60 days, the median tumor volume of cisplatin-treated mice was 225 mm^3, whereas the median tumor volume of photoactivated OCTBP-treated mice was 250 mm^3.

The data for each individual mouse in the photoactivated OCTBP and cisplatin groups are presented in (Fig. 2di and ii). In the photoactivated OCTBP-treated group, five mice were clustered with similar tumor volumes between 100 and 200 mm^3, three mice had large tumors (300–700 mm^3; Fig. 2di), and two mice died during UV irradiation. Otherwise, no morbidity or mortality was observed due to treatment. In the cisplatin-treated group, two mice had tumor volumes of 150–250 mm^3, two had large tumors (300–450 mm^3), two were 'cured', and two mice died during the experiment due to drug toxicity (Fig. 2dii). All the cisplatin-treated mice, including the cured mice, lost approximately 20% of their original weight and looked morbid.

In Fig. 3a and b, we show the tumor growth delay, that is, the median time needed for an individual tumor to reach 500 mm^3. It took 16 more days for the tumors in mice treated with OCTBP to reach this volume compared with 10 days for the tumors in mice treated with cisplatin.

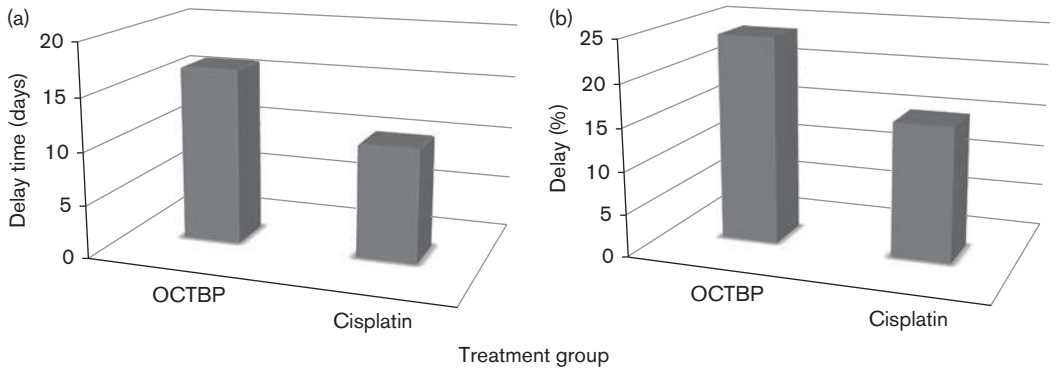
Effects of *cis*-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride in inducing apoptosis in SKMEL-5 xenografts

The apoptotic index was determined in tissues from each group as above. Previous studies and reports in the literature have indicated that a meaningful change in apoptotic index is defined as at least a doubling of the index relative to controls [12]. In fact, we estimated that the apoptotic index approximately more than doubled in the photoactivated tumor-bearing mice compared with the light-only and drug-only groups (Fig. 4a–c and h).

Cell proliferation

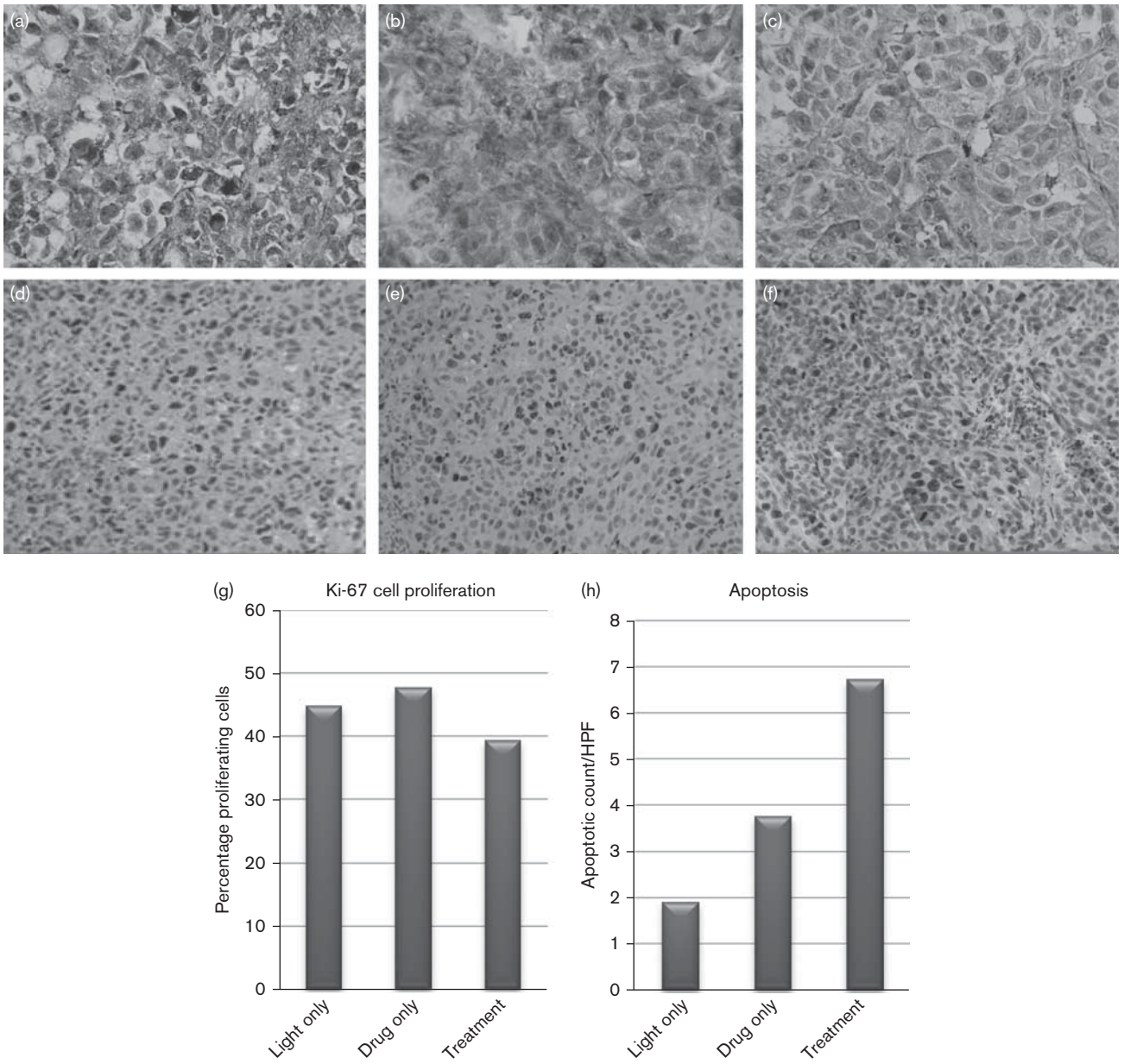
Immunostaining for Ki67 protein, which is associated with cellular proliferation, was performed on tumor tissues from tumor-bearing mice in the untreated group and from the light-only, drug-only, and treatment groups.

Fig. 3



Calculated median time needed for an individual tumor to reach 500 mm^3. Sixteen days needed for the individual tumors in the photoactivated *cis*-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride (OCTBP) group compared with 10 days needed in cisplatin-treated group to reach the 500 mm^3volume (a). (b) Shows the percentage of tumor delay for both groups.

Fig. 4



Photomicrographs demonstrating apoptosis (a–c) and Ki67 immunoreactivity (d–f) in SKMEL-5 xenografts. (a) Photomicrograph from a representative section of tumor treated with photoactivated *cis*-dichlorobis (3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride (OCTBP) xenografts with multiple apoptotic cells shown as brown nuclear staining. (b and c) Photomicrographs from representative sections of tumor treated with light or drug-alone controls showing minimal or no apoptosis detected. Number of apoptotic cells per high-power field for each groups are shown in the bar graph. Staining intensity for Ki67 is comparatively weak in tissue from xenografts treated with photoactivated OCTBP (d) compared with tissue sections from xenografts treated with light or drug alone (e and f). Percentage of cells stained for Ki67 are shown in the bar graphs (g and h).

Ki67-labeled cells were observed in the epithelium in all tumors with fewer proliferating cells observed in the treatment group compared with the light-only and drug-only groups (Fig. 4d–f and g).

Discussion

There is continued interest in the use of PDT in cancer treatment, because the technique conveys exquisite control over both time and place in treating the disease. With appropriate shielding, the drug is activated only at the tumor site, and as the drug is benign in the dark, it is active only when the light is on. However, traditional PDT relies on the formation of reactive oxygen species (ROS) that result in DNA damage and consequently cell death. ROS also result in hypoxia, which may cause some molecular and physiological changes that may lead to

neovascularization and angiogenesis [8], thus allowing tumor cells to grow and invade nearby tissues and become resistant to therapy. Furthermore, melanosomes in melanoma also produce ROS, in addition to those generated by cancer cell metabolism. Together, these activate cellular signal transduction pathways that prevent cell death [14]. However, the rhodium complexes that we have been studying do not depend on oxygen for their mechanism of action. Rather, they specifically target the nucleic acids through the formation of covalent bonds between a base and the metal, in a manner analogous to the mode of action for cisplatin (in fact, we have elsewhere referred to these reagents as 'photocisplatins').

In this study as well as in an earlier study with OCTBP, we observed that this compound is lethal when irradiated in the presence of several cancer cell lines, including Kb, bladder, pancreatic, and colon cells. In addition, we tested the cytotoxic effect of OCTBP on melanoma cells, specifically, the SKMEL-5 and UACC6-2 cells. The drug was found to be phototoxic to both, but the UACC62 cells were susceptible to UV irradiation itself. For SKMEL-5 melanoma cells, minimal cell death was observed in the presence of light only. Moreover, the growth of these cells was effectively reduced upon treatment with photoactivated drug, that is, we observed 40% cell death at a 100-μmol/l concentration of the drug. It is noteworthy that for this cell line there was minimal cell death in the absence of light, a requisite for phototherapeutic specificity.

These in-vitro studies provided the basis for the in-vivo studies, and therefore SKMEL-5 was used for these studies. Before determining the OCTBP efficacy *in vivo* in mice carrying SKMEL xenografts, we determined the optimal delay between drug administration and irradiation and found that it was significantly shorter (10 min) for intravenous injection than for intraperitoneal injection (2 h). Interestingly, no tumor inhibition was seen with a longer time delay of 30 min after i.v. injection, a possible consequence of rapid drug physiological clearance by the mice. In contrast, no tumor inhibition was seen when there was no wait (0 time) after drug injection, because some delay period is needed for the drug to reach, and accumulate in the tumor. We therefore adopted intravenous injection for our protocol, with the chosen dose as the MTD (4 mg/kg) with a 10-min wait time after injection. OCTBP is most readily activated by 311-nm light, although its absorption does extend farther into the long-wavelength UV (studies are ongoing to determine OCTBP efficacy in inhibiting tumor growth at these wavelengths). UV light may cause skin injury independent of interaction with a drug. This is especially the case for athymic mice or SCID mice as they have thin skin with no hair or pigmentation. We therefore determined the effect of 311-nm light on the skin in a pilot study before beginning the efficacy studies. Exposing the mice to light for as much as 30 min produced no injury to the skin as determined by the absence of erythema. Thus, the complete protocol for the murine studies involved the administration of OCTBP at a dose of 4 mg/kg, a delay of 10 min, and then irradiation with 311-nm light for 30 min. It should be noted that we also used multiple drug injections over the course of the treatment. Five injections, given 3 days apart, were determined in our early studies to be well tolerated by the mice. The rationale for a multiple-dose regimen is to target any cells that escaped death at the first treatment, as well as to act on newly growing cells. This is also true in a previous study, in which we found that cisplatin was well tolerated by athymic mice, a three-dose regimen of 0.6 mg/kg of cisplatin given 1 week apart (unpublished data). As cisplatin is a common chemotherapeutic drug that is often used for the treatment of melanoma, its effectiveness in inhibiting melanoma growth was used in these studies as a reference to which we compared the effectiveness of photoactivated OCTBP in inhibiting melanoma growth.

The use of photoactivated OCTBP resulted in an almost 50% tumor inhibition compared with vehicle and drug controls (Fig. 2b) at the end of the 60 days experimental period. Interestingly, there was some indication of inhibition of tumor growth by the drug in the dark, which needs to be confirmed in follow-up experiments (data not shown); this effect had disappeared by the end point of the experiment. It is worth mentioning that the mice were kept in 12 h of light/dark cycles and the dark tumor inhibition seen may be due to the effect of visible light on OCTBP.

The majority of the mice in the treatment group (OCTBP plus light) carried similar tumor volumes (100–200 mg); three mice developed large tumors (300–700 mg). The outliers could simply be the result of a variation in how fast mice clear the drug, or a consequence of the technical challenge in injecting identical amounts of the drug using the (quite small) tail vein.

Apoptosis analysis, using the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, showed that there was an increase in the number of apoptotic cells in tumor tissues in mice treated with photoactivated OCTBP. This was not observed in tumor tissues from mice treated with the drug in the absence of light. The observed doubling of the apoptotic index is consistent with a 50% inhibition of tumor volume. Although there were no significant differences in the numbers of proliferating cells between the control and the drug/light-treated groups, the overall amount of proliferating cells was less in the latter case. It is worth mentioning here that the samples for apoptosis and immunohistochemistry were obtained at the end of the experiment.

We have not determined the extent to which there is any selective drug accumulation in normal and melanoma tissue in this study. However, no histopathological differences were observed between tissues from the drug only (no

light) and the drug/light groups. In earlier studies, using OCTBP i.p. administration, no pathological changes or necrosis were seen in normal tissues, including the kidneys, heart, and lung (data not shown).

With the development of advanced technology for cancer screening and the awareness of the public about the benefit of early detection, drugs that are effective at early stages of the disease will augment the screening effort and increase survival rates. When we compared the treatment-relative effects of OCTBP and cisplatin on melanoma tumors, OCTBP was found to be more effective in reducing tumor size at an early stage and achieved a greater tumor delay. The enhanced tumor delay appears to be a consequence of the development of drug resistance toward cisplatin, a phenomenon less apparent with OCTBP therapy (Fig. 2). It is particularly noteworthy that the mice treated with OCTBP phototherapy showed no morbidity or weight loss, whereas several of the cisplatin-treated mice experienced severe morbidity and variation in treatment response that led to killing of some of the animals before the end of the experiment.

In conclusion, our study has demonstrated the phototoxicity of OCTBP toward melanoma, *in vitro* and *in vivo*. Light-activated OCTBP results in significant tumor reduction and tumor growth delay in tumor-bearing mice. This antitumor activity is closely associated with the induction of apoptosis. Additional studies are needed to determine the specific part of the apoptotic process affected. With new techniques becoming available for the early detection of cancers, the use of photoactivated OCTPB may provide a new, nontoxic therapy to prevent progression of this disease. In addition, a combination therapy involving OCTBP phototherapy and traditional chemotherapy may provide a synergistic effect in treating this cancer. Such studies are in progress.

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Conflicts of interest

There are no conflicts of interest.

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