Evaluation of the New Photosensitizer Tookad (WST09) for Photodynamic Vessel Occlusion of the Choroidal Tissue in Rabbits

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PURPOSE. To determine the efficacy of Tookad (WST09; Negma-Lerads, Magny-Les-Hameaux, France) photodynamic therapy (T-PDT) by evaluating the angiographic and histologic closure of choroidal vessels at different radiance exposures, drug dosages, and intervals between photosensitizer injection and laser application in a rabbit model.

METHODS. Chinchilla Bastard rabbits were injected intravenously with three different dye concentrations (2.5, 5, and 10 mg/kg) before application of light. In every group T-PDT was performed at four different times after injection: 5, 15, 30, and 60 minutes with different radiance exposures ranging from 200 to 3 J/cm². Fundus photographs and fluorescein angiograms were obtained 90 minutes after injection. Follow-up angiographies were performed at days 1, 3, 7, and 14 after initial treatment. Histology was performed in selected cases immediately after treatment and on days 1, 3, and 7.

RESULTS. Immediately after irradiation, most of the visible lesions were angiographically hyperfluorescent due to damaged vessel endothelium and associated RPE damage. Lesions from high-radiance exposures revealed immediate hypofluorescence, indicating vessel closure. Hypofluorescent lesions appeared mainly during day 1 (all lesions angiographically visible, some hypofluorescent) to day 3 (all lesions hypofluorescent) after treatment. At day 7, ophthalmoscopically visible hyperpigmentation took place in all lesions. ED₅₀ thresholds for angiographic hypofluorescence determined at day 3 after treatment with 2.5 mg/kg were 18.8 J/cm² (5 minutes), 62.0 J/cm² (15 minutes), and 100 J/cm² (30 minutes); with 5 mg/kg, 8.4 J/cm² (5 minutes), 22.8 J/cm² (15 minutes), 54.5 J/cm² (30 minutes), and 100 J/cm² (60 minutes); and with 10 mg/kg, 11.7 J/cm² (30 minutes) and 54.1 J/cm² (60 minutes). Histology of the angiographically hypofluorescent lesions revealed vessel thrombosis in all groups 1 hour after PDT up to 7 days after treatment. Sparing of photoreceptors indicated selectivity of T-PDT; however, slight damage was partly observable. After 7 days, localized proliferation of the RPE cells was noted and was enhanced 14 days after treatment.

CONCLUSIONS. T-PDT has the potential to achieve selective choroidal vessel occlusion with proper parameter selection, such as (1) 2.5 mg/kg, 5 minutes, 100 J/cm²; (2) 5 mg/kg, 5 minutes, 25 J/cm²; or (3) 5 mg/kg, 15 minutes, 50 J/cm²; however, slight damage to the photoreceptors cannot be ruled out. RPE proliferation indicates primary RPE damage due to PDT, also described with the use of all other photosensitizers. (Invest Ophthalmol Vis Sci. 2006;47:5437–5446) DOI:10.1167/iovs.06-0532

Photodynamic therapy (PDT) involves intravenous injection of a photosensitizer that accumulates in neovascular and tumor tissue. By irradiating the photosensitized tissue with light at the absorption maximum of the dye, cytotoxicity can be achieved.1,2 For neovascular age-related macular degeneration (AMD), which is the leading cause of blindness in patients older than 65 years in the industrialized nations,3–6 PDT using verteporfin has been widely thought during the past years to be successful in preventing visual loss.7 Current approved PDT patterns for treatment of neovascular AMD involves the injection of benzoporphyrin derivatives (BPDs; verteporfin) and irradiation with 689 nm for 83 seconds 15 minutes after injection (600 mW/cm²; 50 J/cm²), which shows the best clinical results for predominantly classic lesions.8 Currently, numerous second-generation photosensitizers have been tested for treatment of neovascular AMD9–11, however, some disadvantages of each of these photosensitizers remain.

The photosensitizer used in this study was bacteriochlorophyll (Bchl; Tookad, [WST09]; Negma-Lerads, Magny-Les-Hameaux, France), a lipophilic, water-soluble derivative of the natural pigment Bchl with an incorporated palladium (Pd) atom and a maximum excitation peak of 762 nm (high-absorption coefficient of 10⁵).12–16 (Fig. 1). Further characteristics of the dye are molecular weight of 714, purity > 95%, stability in air, low rate of photobleaching, and additional spectral peaks at 530, 385, and 330 nm. After intravenous administration, Tookad becomes mainly bound to HDL and LDL proteins. It has an extremely strong vascular effect. The quantum efficiency for triplet state production is approximately 99%, with very high production of singlet oxygen, the putative toxic agent in PDT (type II mechanism). Further studies on the photochemistry of Tookad have revealed the highest photodynamic effect shortly after injection,15 which seems to be due to ultrafast electron transfer from one Bchl to another in loosely coupled dimmers. The anion can reduce the oxygen presence, starting a chain of events ending with the ejection of the hydroxyl radical, an anion in a non-Fenton reaction.15 Because the radicals seem to be ejected directly from the excited sensitizer, it is suspected that their formation falls into neither a type I nor II process but represents a class by itself: a type III process.15

Clinically, the main advantages of Tookad in comparison with other tested sensitizers are as follows: (1) Because of its high-wavelength absorbance, the exciting light beam is able to penetrate deeper into tissues and thus may permit an enhanced selective treatment of the choroidal vessels behind the strong absorbing RPE. (2) Because of its strong optical absorption, excitation with low-energy light sources is possible. (3) A short
delay between injection and irradiation causes mainly vascular
damage. (4) The action of Tookad is rapid (phototoxicity >200
times stronger than with Photofrin II; Axcan, Mont Saint Hi-
lare, Quebec, Canada), with treatment being completed
within 1 hour, and it clears rapidly from the blood circulation
(<24 hours in mice).14 This feature permits ambulatory treat-
ment and presents a low risk of adverse complications such as
photosensitizing of the skin after treatment. (5) Animal exper-
iments show no drug toxicity (in the dark) at a dose 100 times
higher than the effective treatment dose.14
The purpose of this study was to determine the ability of
Tookad to produce choroidal vascular occlusion when different
treatment parameters were used in a rabbit model.

MATERIAL AND METHODS
Lasers
PDT lesions were performed with an argon, pumped-wavelength tu-
neable ti:sapphire laser. The wavelength was continuous wave (cw)
762.0 ± 0.1 nm (full width at half maximum [FWHM] 0.2 nm). The
laser beam was delivered to a clinically used ophthalmic laser slit lamp
(model 1667/88; Carl Zeiss Meditec, GmbH, Oberkochen, Germany)
using a multimode fiber (160 μm, 0.1 NA). The magnification of
coupling was sixfold. The laser beam was focused onto the retinal
surface in the central area of the rabbit eye by a contact lens (Gold-
mann 903; three mirror; Haag Streit, Koniz, Switzerland). With the use
of a magnification factor of 0.66 when irradiating rabbit’s eyes with a
plano-concave contact lens in cycloplegic emmetropic eyes,17 the
retinal spot size appeared to be 634 μm. Irradiation was performed
with 600 mW/cm² and an intensity of 1.89 mW ± 10%. The aiming
laser emitted at 635 nm. To avoid activation of the photosensitizer
due to illumination, we integrated a blue filter (BP459) into the slit lamp.

Ophthalmoscopically visible marker lesions are necessary for the invisible PDT lesions, to demarcate the treatment area and were cre-
ated by the use of an arc-lamp–excited, intracavity, frequency-doubled
Nd:YLF laser (model 527DPH; Quatronix, Inc., Akron, OH), emitting at
a wavelength of 527 nm.18 Suprathreshold irradiation with pulse du-
rations of 1.7 μs (30 pulses, 100 Hz) were achieved. The energy was
transmitted by a 105-μm core diameter fiber (Opttran UV-A 105/125/
250, NA 0.1; Ceram Optec GmbH, Bonn, Germany), which was directly
coupled to the slit lamp fiber (diameter, 158 μm; NA 0.1; Carl Zeiss
Meditec, Inc.).

Photosensitizer
Tookad (bacteriochlorophyll; Bchl) is a water-soluble derivative of the
natural pigment bacteriochloro-
phyll. A Palladium atom is incorpo-
rated within the central part of the
molecule. The peak with the highest
absorption coefficient is at 762 nm.
There are additional peaks at 530,
385, and 350 nm.

Animals
Chinchilla Bastard rabbits were used for the experiments. Each animal
weighed between 2.0 and 3.0 kg. Rabbits were chosen because the
density and location of light-absorbing pigments in the fundus are
rather uniform and similar to that in the human eye.20 The animals
were anesthetized with ketamine hydrochloride (35 mg/kg of body
weight) and xylazine hydrochloride (5 mg/kg of body weight). Pupili-
ary dilatation was achieved with topical application of phentolamine
hydrochloride 2.5% and tropicamide 1%. The animals were placed in a
special holder system that allowed movement in all directions. The
plano-concave contact lens was placed on the mydriatic eye with
methyccellulose used as a contact gel. The lens was locked in the
animal holder to prevent movement. After administration of the dye,
the animals were kept in dark conditions for 3 days. Usually they were
housed in rooms with ordinary fluorescent lamps (12 hours on, 12
hours off). Enucleations were performed with the animals under deep
anesthesia, after which they were euthanatized by an intravenous
injection of pentobarbital sodium.
The treatment of experimental animals in this study was in com-
pliance with the ARVO Statement for the Use of Animals in Ophthalmic
and Vision Research.

Laser Treatment and Documentation
The particular nonvisible PDT lesions (n = 16 per eye for threshold
evaluation) were placed between the marker lesions (n = 6 marking a
field of approximately 6 × 6 mm in the central retina) enabling
orientation during treatment. For the threshold experiments, 24 eyes
were irrigated. Animals were separated into three groups with
Tookad concentrations of 2.5 mg/kg (group 1), 5 mg/kg (group 2), and
10 mg/kg (group 3). In the first 15 eyes (5 eyes per group), radiance
exposures of 100, 50, 25, and 12 J/cm² were applied at each of 5, 15,
30, and 60 minutes after dye injection. A typical irradiation pattern
is shown in Fig. 2. In another nine eyes, irradiation patterns were slightly
changed including 200, 6, and 3 J/cm² for the preliminary calculated

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**Figure 1.** Chemical appearance and excitation spectrum of Tookad, which is a water soluble derivative of the natural pigment bacteriochloro-
phyll. A Palladium atom is incorporated within the central part of the molecule. The peak with the highest absorption coefficient is at 762 nm. There are additional peaks at 530, 385, and 350 nm.
thresholds to gain more lesions at the supposed vessel occlusion threshold. Thus, for each of the single parameters ($n = 48$) at least 5 and at most 14 lesions could be evaluated.

Ninety minutes after application of the laser lesions, fundus photographs were taken with a fundus camera (Carl Zeiss Meditec GmbH). Afterward, standard fluorescein angiography was performed, with injection of 10% fluorescein sodium (2 mL bolus) into the ear vein.

For evaluation of presumed vessel occlusion, the angiographic hypofluorescence in each PDT lesion was plotted versus the laser energy for the various fluences. From the plot, the mean threshold radiance exposures for the different fluences were determined. The angiographic threshold irradiation ($ED_{50}$) was defined as the irradiation necessary to achieve fluorescein angiographically hypofluorescent vessel closure with a 50% probability. For presentations of the $ED_{50}$ the $y$-axis was plotted as an inverse normal distribution function (probability plot) versus the logarithmically calculated energy, resulting in a line of $y = ax$. The thresholds were calculated using software for probit analysis. It should be noted that angiographic hypofluorescence may only signal possible vessel occlusion, which can be verified only by subsequent histology. Also, edema may block choroidal fluorescence leading to hypofluorescence. However, hypofluorescence was often seen without edema and was additionally proven by histology; thus, hypofluorescence could be regarded as a strong indicator for choroidal vessel occlusion.

**Histology**

Based on the obtained threshold results, selected laser parameters in groups 1 (2.5 mg/kg) and 2 (5 mg/kg) were chosen to be evaluated histologically. For this purpose, suprathereshold lesions were placed in a suitable irradiation pattern consisting of $2 \times 3$ lesions per parameter per eye ($n = 12$; Fig. 3). Fluorescein angiography was performed at day 1 after treatment to verify vascular thrombosis for each lesion, as judged by angiographic hypofluorescence. Histology was performed at 1 hour, and 1, 3, and 7 days after irradiation. Immediately after enucleation, the globes were incised anterior to the equator and immersed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer. The posterior eye cup was cut from the anterior segment after 10 minutes and replaced in the fixative. The retinas were then fixed in 2.5% glutaraldehyde and postfixed in Dalton’s osmium fixative, dehydrated in alcohol, and embedded in epoxy resin (Epon). Ultrathin sections were stained with uranyl acetate. One-micrometer serial sections were cut until the center of the lesion was reached. The status of occlusion of the choriocapillaries and the choroid was investigated, as well as the collateral damage of the RPE and the photoreceptors.

**RESULTS**

**Angiographic Evaluation**

Angiography was performed at 1 hour, and 1, 3, 7, and 14 days after irradiation. Usually, at 1 hour most of the lesions that were detectable by angiography revealed hyperfluorescent leakage; however, at high radiance exposures ($100 \text{ J/cm}^2$) initial hypofluorescence indicating vessel occlusion was also detected. After 1 day, more lesions were detectable (previously ophthalmoscopically invisible lesions became visible after 1 day), and most of them were hypofluorescent, but some hyperfluorescent lesions at lower radiance exposures were also observed. At day 3, all detectable lesions were hypofluorescent. Hypofluorescence was stable up to day 7; however, some changes in angiographic patterns were noted that were caused by ophthalmoscopically visible hyperpigmentation, which

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Early angiography revealed hypofluorescence, suggesting choroidal vessel occlusion in three lesions (5 minutes: 100 and 50 J/cm²; 15 minutes: 100 J/cm²). Late-phase angiography revealed additional damage by leakage in three other lesions. In the fundus photograph a slight whitening of two lesions is visible. Early angiography phases revealed hypofluorescence suggesting choroidal vessel occlusion in the first three lesions of the first three rows. Additional leakage was found for the 12 J/cm² lesions in the first three rows and additionally for 100 and 50 J/cm² in the fourth row. Also notable was that the fast pooling effect in the irradiated area enhanced whitening with all but one parameter (12 J/cm², 60 minutes after dye injection). Early angiography phases revealed hypofluorescence suggesting choroidal vessel occlusion in all but two lesions (25 and 12 J/cm², 60 minutes after dye injection). Fast pooling was still present, as seen in the late-phase angiography. Fundus photograph shows light lesions, with especially the first two lesions in the first three rows being very pronounced. Early angiography and fundus photograph 7 days after irradiation in the same eye. The fundus photograph reveals enhanced hyperpigmentation over the whole area, with corresponding angiographic hypofluorescence.
TABLE 1. Efficacy of Tookad at Different Time Intervals between Injection and Irradiation in Rabbits That Had Received 5 mg/kg

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Efficacy</th>
<th>Lesions</th>
<th>Radiance</th>
<th>Damage</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Minutes</td>
<td>100%</td>
<td>2/2</td>
<td>25 J</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>15 Minutes</td>
<td>100%</td>
<td>2/2</td>
<td>50 J</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>100%</td>
<td>2/2</td>
<td>100 J</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>60 Minutes</td>
<td>100%</td>
<td>2/2</td>
<td>150 J</td>
<td>0%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 1 shows the efficacy of Tookad in terms of irradiation at different times after injection as determined at day 3 after treatment (5 mg/kg dye concentration), when PDT effects resulted in maximum angiographic hypofluorescence. Irradiation took place 15 minutes after injection. Hypofluorescent lesions were noted that had no effect on the RPE. However, it was noted that hypoperfusion was enhanced when radiation exposure was high threshold power levels (Fig. 4).

Efficacy of Tookad Regarding Different Time Intervals between Injection and Irradiation

Table 2 summarizes Tookad’s efficacy at various dosages and radiance exposures, as determined by fluorescein angiography at day 3 after treatment. In group 1 (2.5 mg/kg), vascular occlusion was achieved at calculated ED50 thresholds of 22.8 J/cm2, when irradiation took place 5 minutes after dye injection. Irradiation 15 minutes after injection led to a higher ED50 threshold at 62.0 J/cm2. The ED50 threshold for the 30-minute time interval was 11.7 J/cm2, and for 60 minutes it was 54.1 J/cm2.

Histology

Based on the ED50 results for feasible clinical treatments, histology was performed for treatment with 2.5 and 5 mg/kg Tookad. Twelve specimens were examined histologically according to the parameters in Table 3. For treatment with 5 mg/kg dye concentration, histology was evaluated 1 hour and 7 days after treatment (irradiation 15 minutes after injection with 50 J/cm2 and 30 minutes after injection with 100 J/cm2). For both irradiation patterns, 1 hour after treatment the lesions were ophthalmoscopically nonvisible, and angiography revealed focal hypofluorescence. In these areas, histology showed undamaged photoreceptors, a thinner RPE layer, and only some focal occlusion of the choriocapillaris and choroidal vessels (Fig. 6). For specimens with the same parameters, 7 days after treatment, angiography showed hypofluorescence and histology revealed occluded vessels and intact photoreceptors (Fig. 7). Most striking is the finding of proliferating RPE with extensive nodular proliferations leading to multilayered RPE (Figs. 8, 9). Proliferation was more intense when irradiation at 100 J/cm2 was applied 30 minutes after dye injection.

Regarding 2.5 mg/kg Tookad, irradiation at 5 and 15 minutes after dye application (each 100 J/cm2) was examined. One hour after treatment, both parameters revealed histologically significant occlusion at the choriocapillaris.

Table 2. Threshold Power Levels Needed for the Laser Parameters Used to Achieve Angiographically Determined Vessel Occlusion of the Choriocapillaris as Evaluated at Day 3 After Treatment

<table>
<thead>
<tr>
<th>Drug Concentration</th>
<th>Power Levels (J/cm2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mg/kg</td>
<td>8.4 J/cm2</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>22.8 J/cm2</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>54.1 J/cm2</td>
</tr>
</tbody>
</table>

Data are the minutes after administration of the drug and the radiance of the laser treatment.
intact photoreceptors and open vessels within the choriocapillaris and choroid but a thinner RPE layer (Fig. 10). The lesions were ophthalmoscopically invisible, and angiography did not show any hypofluorescence. One day later, lesions were ophthalmoscopically visible as light spots and were angiographically hypofluorescent. Despite these findings, histology showed partly occluded vessels but still some open vessels. The photoreceptor layer was intact, but RPE was dense, thin, and hyperpigmented (Fig. 11). Three days after treatment, the vessels were occluded, slight damage to the photoreceptors was observed, and some proliferation of the RPE was noted (Fig. 12). Histology 7 days after treatment revealed occluded vessels for both parameters, RPE proliferation, and slight damage to the photoreceptors (Fig. 13). No nodular RPE proliferation was noted for the 5-mg/kg dye injection, compared with the 5-mg/kg dye injection.

**DISCUSSION**

Today, just one photosensitizer, verteporfin, is approved for use in clinical treatment of neovascular AMD. However, there are several photosensitizers currently being tested in experimental and clinical studies. To take some advantages in contrast to other sensitizers. One is strong absorption at 770 to 780 nm in the infrared-A range, which enables greater tissue penetration. In addition, Tookad overcomes many of the disadvantages especially of the hematoporphyrin derivatives, because it has been shown to be approximately 200 times more phototoxic and to clear much more rapidly from tissues. The purpose of this preclinical study was to evaluate Tookad’s potential for occlusion of the choriocapillaris vessels in the eye. Angiographic evaluation was performed at five different times throughout the study. It was observed that early changes as determined 1 hour and 1 day after treatment are not sufficient to indicate final vessel occlusion, because more lesions were detectable at 1 day than at 1 hour after irradiation, indicating a time-dependent biological process induced by PDT. This thrombotic process was faster for high-radiance exposures, as seen for 100 J/cm², but it may have taken at least 1 to 3 days for lower radiance exposures slightly above threshold. Thus, complete vessel occlusion derived from angiographically seen hypofluorescence could not be finally judged earlier than day 3.

**FIGURE 6.** Histology 1 hour after irradiation with 5 mg/kg Tookad, 15 minutes after dye injection with 50 J/cm². Displayed is the transition from healthy tissue (left) to irradiated tissue (right). Note the thrombosis of the big choroidal and small choriocapillary vessels on the right side. The RPE looks regular on the left but thinner on the right side. The photoreceptors look intact. Magnification, ×400.

**FIGURE 7.** Histology 7 days after irradiation with 5 mg/kg Tookad, 15 minutes after dye injection with 50 J/cm². This section throughout the whole lesion revealed occluded vessels and intact photoreceptors; however, on the RPE level, proliferation was observed (left side). Magnification, ×400.

<table>
<thead>
<tr>
<th>Drug Dose (mg)</th>
<th>Days Post Treat</th>
<th>Parameter</th>
<th>Ang. 1 d</th>
<th>Opht. 1 d</th>
<th>Retina</th>
<th>RPE</th>
<th>Chorioc.</th>
<th>Choroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0</td>
<td>50 J</td>
<td>NV 1 h</td>
<td>NV 1 h</td>
<td>Intact</td>
<td>Thinner</td>
<td>Open</td>
<td>Open</td>
</tr>
<tr>
<td>2.5</td>
<td>1</td>
<td>50 J</td>
<td>Light</td>
<td>Intact</td>
<td>Dense, thin</td>
<td>50% occluded</td>
<td>50% occluded</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>3</td>
<td>50 J</td>
<td>Light</td>
<td>Light 50% damage</td>
<td>Prolif.</td>
<td>Presum. occluded</td>
<td>Presum. occluded</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>7</td>
<td>50 J</td>
<td>Light</td>
<td>More prolif.</td>
<td>Occluded</td>
<td>Occluded</td>
<td>Occluded</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td>50 J</td>
<td>NV 1 h</td>
<td>NV 1 h</td>
<td>Intact</td>
<td>Thinner</td>
<td>50% occluded</td>
<td>50% occluded</td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td>100 J</td>
<td>NV 1 h</td>
<td>NV 1 h</td>
<td>Intact</td>
<td>Thinner</td>
<td>50% occluded</td>
<td>50% occluded</td>
</tr>
<tr>
<td>5.0</td>
<td>7</td>
<td>50 J</td>
<td>Light</td>
<td>Light damage</td>
<td>Less prolif.</td>
<td>Occluded</td>
<td>Occluded</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>7</td>
<td>100 J</td>
<td>Light</td>
<td>Light damage</td>
<td>More prolif.</td>
<td>Occluded</td>
<td>Occluded</td>
<td></td>
</tr>
</tbody>
</table>

Evaluated is the choriocapillaris status at different times after treatment and the potential collateral damage to the RPE and retina. Parameters are minutes after drug dose and laser fluence. Days Post Treat., days after treatment; NV, non visible; Occ, occluded; Prolif, proliferation; Ang., angiographic appearance; Opht., ophthalmologic appearance; Chorioc, choriocapillaris; Pigm, pigmented; Presum. occluded, presumed occluded.
leakage and pooling of the fluorescein in the subretinal space, effectuating an angiographic hyperfluorescence. This effect raised difficulties in properly judging hypofluorescent lesions, especially within the first 2 days after treatment, when pooling was very fast and angiography was performed using static images of the camera (Carl Zeiss Meditec).

Ophthalmoscopically, the PDT lesions appeared whitish, presumably due to retinal edema in the first 3 days, more enhanced at day 3 than at day 1. At day 7, the lesions faded, and hyperpigmentation was even more pronounced at day 14. With irradiation much above threshold, as seen for 10 mg/kg Tookad with radiance exposures of 100 J/cm² 5 minutes after injection, occasional distinctive fibrosis was noted in the irradiated area. Because of the hyperpigmentation, a blocking phenomenon was present that suggested angiographic hypofluorescence in part (Figs. 4L, 4M). Thus, possible reperfusion of the capillaries could not be determined exactly in all lesions at this time. Extensive hyperpigmentation, as described herein, is often seen in laser trials in the rabbit retina, because these animals are known to have a predisposition for strong RPE proliferation. It is assumed that no gross proliferation takes place in humans; however, a distinct impact of RPE damage from Tookad (T)-PDT cannot be ruled out in humans, as has also been true of all other photosensitizers.

ED₅₀ thresholds were lowest with irradiation at 5 minutes after dye injection for 10 mg/kg (< 8 J/cm²) followed by 5 mg/kg (8.4 J/cm²) and 2.5 mg/kg (18.8 J/cm²) Tookad. With the 10-mg/kg dosage, significant ED₅₀ levels at tested radiance exposures were obtained primarily after 30 minutes (at 5 and 15 minutes all lesions were positive for vessel occlusion), which means a very high concentration of dye and a long waiting time between dye application and irradiation to avoid overtreatment—problems that may make this dye concentration inadvisable for clinical use. Thus, histologic examination of these parameters was not routinely performed. Regarding 2.5 mg/kg, clinically reasonable results were achieved with irradiation at 5 and 15 minutes after treatment; however, 100% probability of generating vessel occlusion was seen only at 100 J/cm² (5 minutes after injection) which resulted in an irradiation time of 164 seconds, relatively long for clinical use. Finally, a dye concentration of 5 mg/kg seems to be appropriate for Tookad. Reasonable irradiation parameters might be 25 J/cm² 5 minutes after injection or 50 J/cm² at 15 minutes. If these proposed parameters are used, the probability of achieving angiographic vessel occlusion is approximately 100% and thus, irradiation is already considered to be significantly higher than is needed for the ED₅₀ threshold.

Comparing all calculated thresholds, as derived from Figure 5 and Table 2, it is notable that with irradiation with a double
dose of dye or an approximately doubled time interval after dye injection, the threshold increase was always approximately two to three times (except for the 10 mg/kg, 30-minute parameter), which is reasonable and shows that the threshold values were correctly determined (Fig. 14). With the proposed parameters, time intervals between dye application and final laser treatment were short—comparable to the current PDT treatments with verteporfin—which may be a benefit for the patient, because it eliminates waiting time before PDT. These data also showed that the efficacy of Tookad 60 minutes after injection was low in all groups. This finding indicates the fast clearance time of the dye from the blood circulation, which lowers the chance of side effects such as skin burns, which were often seen during the TAP investigations with verteporfin.

Histology revealed the potential of Tookad to occlude the choriocapillaris and the choroidal vessels at 2.5- and 5-mg/kg dye concentrations, clearly seen 3 to 7 days after treatment. The observed delay in achieving vessel occlusion after treatment supports the conclusion that vessel occlusion on a photodynamic basis is a dynamic process that predominantly takes place between the first and third days after treatment, if irradiation is slightly suprathreshold. Different mechanisms for the vascular occlusion due to PDT were discussed; however, the main reason is thought to be a huge release of factor VIII or thromboxin after PDT from the damaged endothelial cells, leading to aggregation of thrombocytes.

Because of this—especially at threshold irradiation—a primary apposition thrombosis may occur at the vessel intima, leading to turbulence in the blood flow and consecutive apposition of material at this site and finally to occlusion of the complete vessel lumen. This process is known to be dynamic, depending on dye concentration, radiance exposure, and time of irradiation. Thus, in our investigation, histology revealed partly open vessels 1 day after treatment, despite successful irradiation, but closure of all vessels 3 days after irradiation (Table 3). Presumed partial reperfusion of the lesions as assumed from the angiographic findings due to blockage by hyperpigmentation could not be confirmed by the histologic examinations; thus, Tookad seems to have a robust potential for achieving proper vessel occlusion.

Besides verteporfin, the other major photosensitizers of the second generation, which have entered preclinical and clinical trials, are SnET2 (tin ethyl etiopurpurin; purlitin), lutetium texaphyrin (Lutex; Alcon, Fort Worth, TX), mono-l-aspartyl chlorine e6 (NP6), and ATX-S10.

Purlitin, a lipophilic sensitizer, photoactivates at 664 nm and occludes choriocapillary vessels successfully in pigmented rabbits when irradiation starts 15 to 45 minutes after dye injection at an irradiance of 300 mW/cm² and relatively light irradiances.
doses of 5 to 20 J/cm². RPE damage and outer retinal alterations were documented using the optimal parameters for vessel occlusion (Moshfeghi DM, et al. IOVS 1995;36:ARVO Abstract 115).\(^\text{10}\) This photosensitizer has already been evaluated in clinical trials with vision results comparable to those of verteporfin therapy; however, treated patients have to avoid bright light for several weeks because of prolonged retention of the sensitizer within the skin.\(^\text{29}\) Moreover, in comparison to Tooak, water solubility is poor, and the extinction coefficient is only a third of Tooak's.\(^\text{30}\)

Lutetium texaphyrin, a water-soluble, synthetic porphyrin analogue, photoactivates at 752 nm, improving tissue transmission due to the longer wavelength.\(^\text{31}\) In an experimental model of laser-induced choroidal neovascularization (CNV) in the monkey, absence of fluorescein leakage from the CNV lesion was obtained with treatment using 2 mg/kg sensitizer and 50 or 100 J/cm² at an irradiance of 600 mW/cm² (Arbour JD, et al. IOVS 1999;40:ARVO Abstract 401).\(^\text{32}\)\(^\text{33}\) Occlusion of the choriocapillary layer was found in all parameters tested; however, damage to the neurosensory layer and necrosis of the RPE was also described (Arbour JD, et al. IOVS 1999;40:ARVO Abstract 401). A clinical trial is under way. Because the extinction coefficient is only a third of Tooak's,\(^\text{30}\) the photodynamic effect is regarded to be less. Human plasma half-lives are brief (0.25–8.8 hours), which is a considerable advantage.\(^\text{30}\)

Another water-soluble photosensitizer in animal studies is ATX-S10, which has selectively occluded experimental CNV in a rat model (Obana A, et al. IOVS 1998;39:ARVO Abstract 389). ATX-S10 photoactivates at 670 nm, and vascular occlusion has been evaluated at various drug doses, irradiances, and onepigmented animals.\(^\text{34}\)\(^\text{35}\) The described side effects were retinal thinning, loss of photoreceptor outer segments, and RPE proliferation.\(^\text{32}\)\(^\text{35}\)\(^\text{36}\) Long human plasma half-lives of 9 to 134 hours are disadvantageous and also the extinction coefficient is considerably lower than that of Tooak.\(^\text{30}\)

Because animal models and parameters used in different photosensitizer studies are heterogeneous, the extent of RPE damage cannot be compared; however, the RPE reaction of Tooak is expected to be significantly lower in human trials. Compared with other photosensitizers, the main advantage of Tooak seems to be photoactivation at the highest wavelength of all sensizers with the best passage into the deeper tissues of the choriocapillaris. Moreover, it has a rapid clearance, leading to fewer side effects such as skin burns, and exhibits the largest extinction coefficient at a factor of 10\(^\text{3}\). However, it is considerably debatable whether higher-wavelength activation really leads to a more selective effect or whether unwanted side effects such as choroidal occlusion are enhanced. Even when longer wavelengths are used, the extremely light-sensitive RPE will be harmed, then producing cytotoxic free radicals released into the cytosol and consecutively damaging the RPE.\(^\text{30}\) The recurrence rate of choroidal neovascularization in clinical application using BPD is approximately 50%.\(^\text{36}\) Because the cellular phototoxicity of Tooak is regarded to be higher than that of other sensizers, it is supposed that the recurrence rate may be decreased in neovascular AMD treated with Tooak.

In summary, T-PDT was first evaluated for opthalmologic concerns in this preclinical study for dose-range determination. It was shown that T-PDT has the potential to achieve choroidal vessel closure. Proper irradiation parameters may favor 5 mg/kg dye concentration and irradiation 5 minutes after dye application at a radiance exposure of 25 J/cm² or 15 minutes after dye application at a radiance exposure of 50 J/cm². Unwanted side effects such as RPE proliferation were observed but have also been described for all other photosensitizers tested so far. Also, slight photoreceptor damage could not be ruled out in all cases. However, due to the high wavelength and deeper tissue penetration of laser light, collateral damage to the neurosensory layer may be less in humans than with other photosensitizers. Thus, human studies are necessary to determine the future role of this agent in the treatment of AMD.

References

rophyll based PDT of solid tumors relies on vascular destruction. Proceedings of the 7th Biennial Congress. The International Photo-
dynamic Association, Nantes, France. 1999.


19. Scherz A, Salomon Y, Fiedor L. Chlorophyll and bacteriochloro-


21. Birngruber R, Hillenkamp F, Gabel VP. Experimentelle und theo-
retische Untersuchungen zur thermischen Schädigung des Augen-


ization and normal retina and choroid up to 7 weeks after treat-


enhanced photocoagulation. Lasers Light Ophtalmol. 1993;5:
157–165.

29. Schmidt-Erfurth U, Hasan T. Mechanisms of action of photody-


32. Mori K, Yoneya S, Ohta M, et al. Angiographic and histologic effects of fundus photodynamic therapy with a hydrophilic sensi-
tizer (mono-L-aspartyl chlorine e6). Ophtalmology. 1999;106:
1384–1391.


34. Obana A, Gohto Y, Kaneda K, et al. Selective occlusion of the choroidal neovascularization by photodynamic therapy with a wa-
tersoluble photosensitizer, ATX-510. Lasers Surg Med. 1999;24:
209–222.

2645.

36. Okunaka T, Eckhauser MC, Kato H. Correlation between photo-
dynamic efficacy of different porphyrins and membrane partition-