Photodecomposition, photomutagenicity and photocytotoxicity of retinyl palmitate under He–Ne laser photoirradiation and its effects on photodynamic therapy of cancer cells in vitro

Tarek Ibrahim a, *, Mahmoud N. El Rouby b, El-Sayed A.M. Al-Sherbini a, Amr H. El Noury a, Mona E. Morsy a

a National Institute of Laser Enhanced Science (NILES), Cairo University, Cairo, Egypt
b National Cancer Institute (NCI), Cairo University, Cairo, Egypt

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Objective: Our aim was to study photodecomposition, photomutagenicity and cytotoxicity of retinyl palmitate (RP), a principal storage form of vitamin A in humans and animals, under He–Ne laser photodecomposition. Moreover, the effect of different concentrations and timing protocol of antioxidants on photodynamic therapy (PDT) is contradictory, so the effect of RP (as antioxidant) on the PDT cytotoxicity was studied.

Methods: Photomutagenicity was tested by Ames test. Photodecomposition was studied by UV–vis spectroscopy. Cytotoxicity was measured with MTT-assay. Moreover, the effect of PDT, using hematoporphyrin derivatives (HpD) as photosensitizer under He–Ne laser irradiation (100 J/cm²), was studied on HeLa cells either with or without RP (1–100 μM) which incubated with the cells for short or long incubation period (1 h or 24 h) prior to PDT.

Results: No photodecomposition of RP alone was observed whereas there is a little photodecomposition of RP only in presence of HpD under irradiation with He–Ne laser. Moreover, no photomutagenicity was observed in Salmonella typhimurium strains under laser irradiation in presence or absence of HpD. RP alone (1–100 μM) significantly decrease the viability of HeLa cells. Laser irradiation of HeLa cells pre-incubated with RP alone for 24 h showed further significant decrease in viability of the cells. While RP incubations for 1 h before PDT had slight effect on the cells, 24 h incubation before PDT enhanced the cytotoxicity of PDT on HeLa cells.

Conclusions: RP can be used 24 h before PDT to enhance its effects. RP is not mutagenic under irradiation with He–Ne laser.

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1. Introduction

Vitamin A (retinol) and its derivatives, collectively named retinoids, are required for many biological processes including cell growth, differentiation and maintenance. Retinoids are comprised of three units: a bulky hydrophobic region, a linker unit and a polar terminus which is usually a carboxylic acid. Modification of each unit has generated many more compounds [1]. Retinyl palmitate (all-trans-retinyl palmitate, RP) is a principal storage form of retinol in humans and animals that can be enzymatically hydrolyzed back to retinol in vivo. RP is widely used because retinol is thermally unstable while RP is relatively more stable [2, 3]. Retinoids inhibit tumor formation and skin cancer development in experimental systems and in humans. Retinol is described as an inhibitor of cell growth in G1 phase. There is good evidence that the antitumor activity of retinoids is partially due to induction of cellular differentiation and/or inhibition of cell proliferation. Retinoids inhibit the proliferation of cells associated with HPV infection. They also have promising effects in inhibiting the progression of early cervical lesions to cancer [4].

The clinical evidence for a retinoid-based clinical chemopreventive approach has originated from the successful retinoids treatment of premalignant lesions such as oral leukoplakia, cervical dysplasia and xeroderma pigmentosum. Clinical trials revealed that retinoids are active in reducing some second primary cancers
such as aerodigestive tract tumors, second primary lung cancers and second hepatocellular carcinomas [5].

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) [6]. There are various types of retinoid-binding proteins which are located in intracellular and extracellular compartments and associated with isomeric forms of retinoids. Hence, retinoids are either associated with cellular membranes or bound to a specific retinoid-binding protein. These binding proteins solubilize and stabilize retinoids in aqueous spaces and they, along with nuclear receptors, mediate the action of retinoids [1].

Retinol and RP are considered safe as they are included in the Generally Recognized as Safe Substances Database (GRAS). Hence, they are found in cosmetic products, food and dietary supplements. However, the safety of topical retinoids was questioned in one publication and in a recent National Toxicology Program Report [7]. These reports suggested possible photomutagenicity of retinoids under ultraviolet irradiation. This suggestion contradicts a large body of data indicating that topical retinoids are chemoprotective in humans. Furthermore, these reports were immediately challenged by new reviews on the safety of RP in general and within sunscreens [8].

Photodynamic therapy (PDT) is a treatment for cancer and certain non-cancerous diseases that are generally characterized by overgrowth of unwanted or abnormal cells. PDT kills cancer cells by generation of reactive oxygen species following absorption of visible light by photosensitizers. These photosensitizers are characterized by high quantum yield to generate highly reactive cytotoxic singlet oxygen only in the presence of coherent and incoherent light [9].

Ames test is a bacterial reverse mutation assay used to test mutagenic properties of chemical compounds. This assay is carried out using several histidine dependent strains of bacteria (Salmonella typhimurium). Each tester strain contains a different type of mutation in the histidine operon that greatly increases their ability to detect mutagens. When the Salmonella tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen

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**Fig. 1.** UV–vis spectrum of retinyl palmitate (RP) alone before irradiation — and after irradiation . . . . . . . with red He–Ne laser (10 J/cm²), no photodecomposition takes place under these irradiation settings.

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**Fig. 2.** UV–vis spectrum of retinyl palmitate (100 µM) mixed with HpD (25 µg/ml) in a ratio of 1:1 v/v before irradiation (−) and after irradiation (⋯⋯) with red He–Ne laser (10 J/cm²). Inset shows a part of UV–vis spectrum of HpD to show the range of visible absorption peaks and shoulders in the red spectrum which are utilized for PDT treatment. Note that the degradation after laser irradiation takes place only in the UV range corresponding to the curve of RP which may be due to the free radical formation as a result of the photosensitization process.
is added to the plate, the number of revertant colonies per plate is increased in a dose-related manner \cite{10}.

The aim of this study has been to investigate the photocytotoxicity, photodecomposition and photomutagenicity of RP under He–Ne laser photoirradiation. Moreover, the effect of antioxidants such as vitamin A on PDT is contradictory and a few studies have been carried out to discuss the role of retinoids in combination with PDT. Hence, the effects of different concentrations and timing protocols of RP on the PDT cytotoxicity were studied.

2. Materials and methods

All chemicals were obtained from Sigma (USA) unless otherwise indicated.

2.1. Cell line

The human cervical carcinoma cell line (HeLa) was obtained from The Holding Company for Biological Products & Vaccines (VACSERVA), Cairo, Egypt and maintained in RPMI-1640 medium containing 100 U/ml Penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO₂ in air at 37 °C.Logarithmically growing cells with at least 80% confluency were used for all experiments.

2.2. Bacterial strains for Ames test

Four strains of histidine requiring \textit{S. typhimurium} LT2 bacteria (TA 97, TA 98, TA 100 and TA 102) were used for the study. They were obtained as a gift from Virology and Immunity Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. Periodic testing revealed appropriate sensitivity to ampicillin and crystal violet. Each test strain carries a different type of mutation (defect) in the histidine operon. They were lyophilized and when dissolved, they were aliquoted and stored at −80 °C until used \cite{10}.

2.3. Preparation of HpD

A stock of Hematoporphyrin derivatives (HpD) was prepared by dissolving 672 mg of Hematoporphyrin derivative dihydrochloride (Sigma–Aldrich, Germany) in 100 ml glycerol by stirring overnight. The stock solution was kept in the dark. Working solutions of HpD (25 μg/ml) were prepared immediately before use by further diluting of the stock solution with a medium without serum, the working solution was sterile filtered with 0.22 μm filter \cite{11}.

2.4. UV–vis spectroscopy

The absorption spectra of RP, HpD and HpD (25 μg/ml) mixed with RP (100 μM) in a ratio of 1:1 v/v before and after irradiation with red visible He–Ne laser were obtained using T80+ UV–vis spectrophotometer (PG instruments Ltd., England) in 1 cm optical path quartz cuvette using UVWIN software version 5.2 (PG instruments Ltd., England). For each UV–vis analysis, a cuvette filled with each sample was undergone UV–vis measurement before laser irradiation (all measurements were made in triplicate). The absorption spectra of other three samples were obtained, after laser irradiation, at different sampling time (1, 3, 5 h post irradiation). Each experiment were repeated three times. All analyses were performed at the National Institute of Laser Enhanced Sciences (NILIES).

2.5. Mutagenicity assay

Thirty milliliter of minimal glucose agar medium (consisting of Vogel-Bonner E medium supplemented with glucose (0.5%, w/v) and agar (1.5%, w/v)) was poured in each Petri dish and left to solidify. For the plate incorporation test, top agar (0.6% agar and 0.6% NaCl) supplemented with histidine and biotin was prepared (containing the tested sample, the bacterial strain and S9 mix in soft agar) as follows: two milliliter of molten top agar supplemented with 0.05 mM histidine and biotin was distributed in 10 ml tissue culture tubes held at 45 °C in water bath and then 0.5 ml of metabolic activation S9 mix was added. Then, we added 0.1 ml of tested samples (RP alone or RP irradiated with laser or mixture of both RP and HpD either kept in dark or irradiated with laser). A fresh overnight culture of the tester strain (2 × 10⁸ bacteria per tube) was added. The contents of the test tubes were then mixed and poured onto the surface of minimal glucose agar plates. Positive and negative controls were also included in each assay. When the top agar had hardened, the plates were inverted and incubated at 37 °C for 48 h. Revertant colonies were counted and the results were expressed as the number of revertant colonies per plate \cite{10}.

2.6. Cytotoxicity of RP on HeLa cells

HeLa cells were incubated with different concentrations of RP (1–100 μM) for 24, 48, and 96 h. After incubation periods, cytotoxicity was evaluated by MTT viability assay.

2.7. Photodynamic treatment

HeLa cells in the exponential phase of growth were harvested and suspended in RPMI 1640 medium with 10% FCS at a density of 40,000 cells/well (2 × 10⁶ cells/ml). Cells were cultured in Microtiter 96 well plates and incubated for 24 h for adhesion. After adhesion, 25 μg/ml HpD was added to each well except untreated controls. The plate was incubated for 24 h in the dark at 37 °C in a humidified CO₂ incubator. Fresh phenol red-free RPMI 1640 medium without FCS was added to each well before irradiation. Cells were irradiated with He–Ne laser (λ = 632.8 nm) at 10 J/cm² for laser treatment. Other plates were left in dark for dark controls.

2.8. Effect of short-term incubation of RP on HeLa cells before PDT

HeLa cells were incubated with HpD for 24 h. Different concentrations of RP (1–100 μM) were added to the cells in the last hour of HpD incubation. The cells were washed three times with sterile phosphate buffer saline (PBS) and fresh media without serum were added to the cells. Cells were irradiated with He–Ne laser. Viability was measured by MTT after 1 h and 24 h post irradiation.

2.9. Effect of long-term incubation (24 h) of RP on HeLa cells before PDT

HeLa cells were co-incubated with HpD and the used concentrations of RP (1–100 μM) for 24 h in the dark. After incubation, the cells were washed three times with PBS and fresh media without serum were added to the cells. Cells were irradiated with He–Ne laser. Viability was measured by MTT after 1 h and 24 h post irradiation.

2.10. Statistical analyses

The statistical analyses were carried out by Graphpad® Prism software (USA). Results were analyzed statistically by the D’Agostino and Pearson test and the Shapiro–Wilks test to ensure normal distribution of data as recommended by the software guide for small number of samples. Then, we carried out on-way ANOVA test followed by Student’s t-test. Values of \( P < 0.05 \) were considered statistically significant. All data in the text and tables are expressed.
Fig. 3. Cytotoxicity of retinyl palmitate (RP) in HeLa cells (graph and table). The cells were treated with different concentrations of RP (1–100 μM) and kept in dark for 24, 48 and 96 h. All tested concentrations of RP significantly decrease the viability of HeLa cells. One interesting observation is that all concentrations cause approximately the same effect which is clearer after 96 h. The table shows that there are a significant decrease in viability after 24 h incubation (which seems overlapped in the graph). The data points represent the mean ± standard error from three independent experiments (n = 12). Statistically significant differences are labeled as *p < 0.05, **p < 0.01, and ***p < 0.001.

### 3. Results

#### 3.1. Photodecomposition of retinyl palmitate

Under red visible He–Ne laser irradiation (632.8 nm, 10 J/cm²), RP alone in the absence of HpD did not have any photodecomposition as shown in Fig. 1. At different sampling time (1, 3 and 5 h post irradiation) there is not any degradation occurred. On the other hand, He–Ne laser irradiation of RP mixed with HpD caused little photodecomposition in the range of the UV spectrum from 210 nm to 385 nm (corresponding to the absorption spectrum of RP); which may be due to the effect of producing free radicals from the photosensitization process of HpD as shown in Fig. 2.

#### 3.2. Photomutagenicity test

We studied the effect of RP on S. typhimurium tester strains TA98, TA100, TA102, and TA104. We found that RP was not mutagenic in S. typhimurium tester strains in the presence or absence of S9 activation enzymes (all colonies formed are less than 20, so data were not shown). Moreover, RP was not photomutagenic in S. typhimurium tester strains upon visible He–Ne laser irradiation (632.8 nm, 10 J/cm²) in the presence or absence of HpD.

#### 3.3. Cytotoxicity of RP on HeLa cell line

All tested concentrations of RP alone (1–100 μM) incubated on HeLa cells for 24, 48 and 96 h significantly decrease the viability of HeLa cells. One interesting observation is that all concentrations cause approximately the same effect, which is clearer after 96 h post treatment as shown in Fig. 3.

#### 3.4. Effect of He–Ne laser irradiation on HeLa cell line incubated with RP

Irradiation of HeLa cells incubated with tested concentrations of RP for 24 h by He–Ne laser at 10 J/cm² showed further decrease in the viability of the cells as compared to the effect of the RP alone in dark group as shown in Table 1. Irradiation of HeLa cells with He–Ne laser increase cytotoxicity by 10 ± 2.5% for each RP concentration as compared to dark RP treated group.

### 3.5. Photodynamic studies

#### 3.5.1. Effect of PDT on HeLa cells

The viability of the cells decreased to 53.26 ± 1.92% (p < 0.001) 1 h post HpD-PDT treatment (HpD 25 μg/ml pre-incubated for 24 h in dark followed by irradiation with He–Ne laser at 10 J/cm²). After 24 h post-irradiation, only 20.44 ± 1.59% (p < 0.01) of the cells survived PDT treatment as shown in Fig. 4.

#### 3.5.2. Effect of short-term incubation (1 h) of RP on HeLa cells before PDT

While short-term incubation of 1 μM RP before PDT caused a little enhancing effect on the cytotoxicity induced by PDT 24 h post irradiation, short-term incubation of 10 and 100 μM of RP (pre-incubated for 1 h before irradiation) had no effect against cytotoxicity of PDT as shown in Fig. 5.

#### 3.5.3. Effect of long-term incubation of RP (24 h) on HeLa cells before PDT

Different concentrations of RP incubated in the cells 24 h before PDT showed that all concentrations used had significant decrease in viability after 24 h post irradiation as shown in Fig. 6.
4. Discussion

Retinoids are recognized as crucial factors in controlling cellular differentiation and exert a wide range of effects on embryonic development. Retinoids also exert functional effects on vision, cell growth, reproduction, apoptosis and resistance to infection in addition to their anti-oxidant effects [1,12]. They have been used in both prevention and treatment of various types of human neoplasms such as cervical cancer and precancerous conditions. Studies described retinol as an inhibitor of cell growth in G1 phase [4].

4.1. Cytotoxicity of RP on HeLa cells

Our results indicate that RP (1–100 μM) has a cytotoxic effect on HeLa cells; the highest decrease in viability was after 96 h of incubation. Moreover, RP seems to inhibit cell proliferation after 48 h incubation as shown in Fig. 3. These results are partially in agreement with that of Maziere et al. [13]. Their results showed that RP, after 1 day of treatment in a rat colonic tumor cell line, decreased cell proliferation, induced cell differentiation and increased the number of cells falling in apoptosis. Our results may be explained by the results of Volz et al. [14] who found that RP concentration was extremely low in CIN III compared to the normal cervical epithelium and HPV-infected tissue. They concluded that a local supplementation of vitamin A might contribute to the prevention of cervical neoplasia [15]. One of the explanations for such effect is that induction or inhibition of cell proliferation seems to be dependent on levels of oxidants/antioxidants in the cell. An enhanced, reducing environment of the cell stimulates proliferation and a slight shift towards a mildly oxidizing environment initiates cell differentiation. A further shift towards a higher oxidizing environment in the cell leads to apoptosis and necrosis. Furthermore, there is a link between increased levels of reactive oxygen species (ROS) and disturbed activities of enzymatic and non-enzymatic antioxidants in tumor cells [16]. It has been reported that, in most sensitive cells, retinoic acid blocks cell-cycle progression somewhere in the G1 phase of the cycle, for example, it arrests human neuroblastoma cells in G1 within 2 days of retinoid treatment. In addition to inducing growth arrest, retinoic acid and various retinoid analogs can induce apoptosis in certain cell types (e.g., human hepatoma cells and a variety of adult T-cell leukemia cell lines). Retinoic acid has the ability to induce differentiation which would prevent an initiated cell from being promoted into a tumor cell [15].

4.2. Photodecomposition of RP under He–Ne laser irradiation

Retinoids are unstable due to the presence of conjugated double bonds and easily undergo oxidation and/or isomerization in the presence of oxidants, light or excessive heat [17]. We studied the photodecomposition of RP under red visible He–Ne laser irradiation (λ = 632.8 nm, 10 J/cm²); no degradation was found in irradiation of RP alone as shown in Fig. 1. However, He–Ne laser irradiation of RP in the presence of HpD caused little degradation in the range of the UV spectrum from 210 nm to 385 nm as shown in Fig. 2. This UV range corresponds to the absorption spectrum of RP, which may be due to the effect of producing free radicals from the photosensitization process of HpD. These free radicals may lead to degradation of RP.

4.3. Photocytotoxicity of RP on HeLa cells

In this study, we found that He–Ne laser photoradiation of HeLa cells, pre-incubated with the tested concentrations of RP for 24 h, caused a little but significant decrease in viability compared to the effect of the RP alone as shown in Table 1. This in part may be explained by the fact that carotenoids and retinoids have conjugated polyene systems that absorb light in the visible and ultraviolet spectrums [17].

One possibility which needs a separate study is that, the effect is due to the production of ROS by laser biostimulation on the cells since the used laser dose is within the biostimulation range (biostimulation wavelength ranges from 600 to 1100 nm, with output power between 1 and 500 mW, and energy density between 0.04 and 50 J/cm²) [18]. The mechanism of laser biostimulation is based on the absorption of monochromatic light by components of the cellular respiratory chain, including mitochondrial NADH-dehydrogenase and cytochrome C oxidase which are among the primary cellular photoacceptors. NADPH-oxidase constitutes a redox chain that generates reactive oxygen species in response to activation and can react to laser irradiation [19]. The production of ROS may cause RP decomposition inside the cells, which did not happen under irradiation in the absence of the cells. When Xia Q and coworkers studied the photodecomposition of RP under ultraviolet and visible light, cell death was observed for retinoid concentrations of 100 μM or higher. They identified its photodecomposition products as 5,6-epoxy-RP or Anhydroretinol (AR). They found that
photoirradiation of RP and its two photodecomposition products generates singlet oxygen and superoxide radical anion that induce DNA damage and cytotoxicity. They concluded that RP and its photodecomposition products can act as a photosensitizer leading to free radical formation and induction of lipid peroxidation following irradiation with UVB light [3,20–23].

4.4. Effect of PDT on HeLa cells

We found that HpD-PDT is effective against HeLa cell line (25 μg/ml HpD, under irradiation with He–Ne laser (632.8 nm) at a total dose of 10 J/cm² and fluence rate of 10 mW/cm²), this concentration and dose agree with our previous work [11]. These parameters are in agreement with that of Wu et al. who studied HpD-PDT on the human lung adenocarcinoma A549 cell line (16 μg/ml HpD, He–Ne laser with the same fluence and fluence rate) [24].

4.5. Effect of RP incubation timing protocol on PDT

Timing of adding antioxidants before PDT is the key element in determining the PDT outcomes. In our previous work, we found that in short incubation time, most of the antioxidants remain in the cell membranes so they are likely to protect or at least decrease the PDT action on cells but after 24 h or long incubation time, they spread in the cell and exert other mechanisms to enhance the effect of PDT [11].

In this study, we found that incubation of different concentrations of RP for short-term incubation (1 h) before PDT showed that high concentrations used have no effect while lower concentration has a little decrease of viability as shown in Fig. 5. On the other hand, incubation of RP for 24 h before PDT showed that all concentrations had a significant decrease in viability after 24 h of irradiation as shown in Fig. 6. This decrease in viability may be an additive effect of the combined treatment of RP and PDT. Another possible reason for the toxicity of reducing agents can arise from the reaction with a photodynamic sensitizer in its excited triplet state (3S), that leads to the formation of the antioxidant and sensitizer radicals that promote type I photodynamic reactions. This reaction has been proposed for electron donors such as ascorbate, cysteine or reduced pyridine nucleotides, but cannot be theoretically excluded for other reducing agents [25].

4.6. Mutagenicity of RP

The role of retinoids on the cytotoxicity induced by PDT is an interesting point and should be studied extensively because retinoids are photodegradable and may cause mutations or DNA strand breaks. This may be true for UV light as studied by Xia et al. [3,20–23]. However, we studied the mutagenicity of RP by Ames test under irradiation with He–Ne laser (with a fluence of 10 J/cm² and a fluence rate of 10 mW/cm²) and the results showed that there is not any mutagenic effect of RP alone, HpD alone or RP mixed with HpD whether in the dark or under irradiation by He–Ne laser irradiation. The lack of mutagenicity of RP in the dark agrees with another study which found that treatment of the LS178Y/TK+/- mouse lymphoma cells with RP alone (25–100 μg/ml) did not increase mutant frequencies over the negative control [3]. These results are very important due to the fact that high doses of individual antioxidant retinoids are being used in the treatment of some human tumors [26]. The timing of RP administration is very important since long incubation time before PDT may have an enhancing effect on the PDT action (compare Figs. 5 and 6).

5. Conclusion

There is not any photodecomposition of RP alone under He–Ne laser irradiation whereas there is a little photodecomposition of RP only in the presence of HpD. No photomutagenicity was observed in S. typhimurium tester strains under laser irradiation in the presence or absence of RpD. RpD alone (1–100 μM) has a significant cytotoxic effect on HeLa cells. Laser irradiation of HeLa cells incubated with RP for 24 h showed further significant decrease in the viability of the cells.

The timing of RP administration is very important since long-term incubation before PDT (24 h) may have an enhancing effect on PDT action as compared to RP incubations for short-term (1 h) before PDT.

References


