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INFLUENCE OF NON-IRRADIATED AND UVA-IRRADIATED L-ARGININE DIPROTOPORPHYRINATE ALONE AND IN COMBINATION WITH 5-METHOXYPSORALEN, ON RESPIRATORY BURST OF HUMAN NEUTROPHILS *IN VITRO*

ANDRZEJ BUGAJ[1], HANNA BILLERT[2], STANISŁAW DYDERSKI[1], ALEKSANDRA KASYNA[1], MONIKA ZBRZEŹNA[1]

[1]Department of Clinical Pharmacy and Biopharmacy, Karol Marcinkowski University of Medical Sciences, ul. Marii Magdaleny 14, 61-861 Poznań, Poland
[2]Department of Experimental Anestesiology, Karol Marcinkowski University of Medical Sciences, ul. Marii Magdaleny 14, 61-861 Poznań, Poland.

The study was focused on the influence of L-arginine diprotoporphyrinate ($PP(Arg)_2$), a new generation photodynamic therapy sensitizer, on respiratory burst of human neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA) and opsonized zymosan (OZ). In 0.5 μM concentration, non-irradiated and UVA-preirradiated $PP(Arg)_2$ did not show any significant effect on luminol-enhanced chemiluminescence of non-stimulated and PMA-stimulated human neutrophils in vitro, except for a weak antioxidative effect of non-irradiated $PP(Arg)_2$ towards non-stimulated cells. Non-irradiated $PP(Arg)_2$ in 0.5 μM concentration significantly decreased chemiluminescence of OZ-stimulated neutrophils; in presence of UVA-irradiated sensitizer, this effect was more pronounced. 0.5 μM $PP(Arg)_2$ in combination with 105 $\mu g \times l^{-1}$ 5-MOP revealed a prooxidative effect towards non-stimulated neutrophils, an antioxidative effect towards OZ-stimulated neutrophils and towards cells stimulated with PMA did not change the luminol-enhanced chemiluminescence. Possible mechanisms of the observed phenomena were also discussed. The obtained results suggest that $PP(Arg)_2$ inhibits EGF-receptor tyrosine kinase whose activity plays an important role in mechanism of respiratory burst stimulation by OZ, differently to analogical stimulatory effect of PMA predominantly connected with release of kinase C. The enhancement of inhibitory effect of $PP(Arg)_2$ after its UVA-preirradiation may be due interaction of excited molecules of senstitzer with superoxide radical anions; the eventual action of irradiation photoproducts could not be also excluded. A synergistic antioxidative effect of $PP(Arg)_2$ and 5-MOP towards OZ-stimulated neutrophils may be a result of addition of porphyrin phototoxic effect and light-dependent antioxidative action of psoralen derivative.

INTRODUCTION

Photodynamic therapy (PDT) is a modality of photochemotherapy, based on the activation of photosensitizing and cell localizing drugs with specific non-ionizing radiation (Dougherty, Gomer, Henderson, Jori, Kessel, Korbelik, Moan & Peng 1998). Molecules of sensitizers excited to triplet states can interact with molecular oxygen to create reactive oxygen species (mainly singlet oxygen) that are thought to cause damage of tumor cells as well as to inhibite the angiogenesis of tumor tissue. This method offers an alternative, less invasive treatment of such diseases as psoriasis and several types of cancer (Oleinick & Evans 1998, Calzavara-Pinton, Szeimies, Ortel & Zane 1996).

The photosensitizers most commonly used in PDT are porphyrin derivatives, such as Photofrin®, approved by U.S. Food and Drug Administration (FDA). As Photofrin® has many important drawbacks (Moan, Peng, Evensen, Berg, Western & Rimington 1987), new strategies of improvement of the efficacy of PDT are needed. Combination of Photofrin® with other photosensitizers demonstrated that 8-methoxypsoralen, a furocoumarin sensitizer applied in PUVA-photochemotherapy (Psoralen + UVA) used in the treatment of T-cell mediated autoimmune diseases such as psoriasis or vitiligo (Potapenko 1991), potentiates the phototoxicity of Photofrin® towards murine tumor cells (Sousa, Maziere, Melo, Vincent-Fiquet, Rogez, Santus & Maziere 1998). On the other hand, a series of other photosensitizers have been proposed to replace Photofrin® (Moan *et al.* 1987; Jori 1996; Brault, Vever-Bizet & Dellinger 1986; Boyle & Dolphin 1996). During the last decade, a new group of photosensitizers has been developed, which are amino acids derivatives of protoporphyrin IX (PPIX). Insertion of endogenous amino acid chain into the vinyl bridge of PPIX compounds might increase its affinity to the membrane recep-

tors in tumor cells while attaching the argininyl cation to the propionic acid groups of porphyrin moiety should enable its solubility in water.

It is also to be into account, that PPIX and its derivatives upon irradiation with light and in presence of oxygen easily forms a variety of photoproducts among these many absorb the same spectral region as PPIX and could also reveal a photodynamic activity (Gudgin Dickson & Pottier 1995). However, another of these products do not absorb light in "therapeutic" region and thus may decrease efficacy of PDT. On the other hand, active photoproduct could reveal phototoxic effect synergistic to PPIX photodynamic action (Gudgin Dickson *et al.* 1995; LiWei, Bagdonas & Moan 2001; Bezdetnaya, Zeghari, Belitchenko, Barberi-Heyob, Merlin, Potapenko & Guillemin 1996).

According to recent studies, neutrophils, the key inflammatory cells involved in resistance of opportunistic pathogens, have become recognized as important contributors to the effectiveness of PDT as engaged in antitumor immune response (Schon & Ruzicka 2001). However, reactive oxygen species released from neutrophils during respiratory burst, may induce permanent mutagenesis resulting in tumor proliferation (Coussens & Werb 2002). Hence, respiratory burst of neutrophils could diminish effect of PDT-treatment (Sun, Cecic, Parkins & Korbelik 2002). On the other hand, PDT-exposition might also modulate respiratory burst of neutrophils, as reported Gal, Kriska and Maltseva (1997) who found decrease of reactive oxygen species (ROS) release from granulocytes treated with red light in the presence of Photofrin (Kal, Kriska & Maltseva 1997). Apart from this, a number of studies concerning interdepence between ROS release and PDT action are strongly limited (Gal, Kriska & Maltseva 1997; Fossel, Fletcher, McDonagh & Hiu 1991).

In the present work, the influence of L-arginine diprotoporphyrinate, $PP(Arg)_2$ - a new, water-soluble porphyrin photosensitizer, on respiratory burst of neutrophils isolated from healthy donors was studied. Following stimulation of neutrophils with the tumor promoter phorbol 12-myristate 13-acetate (PMA) as well as with opsonized zymosan (OZ) it was able to identification. A potential synergistic effect of 5-methoxypsoralen (5-MOP) was also investigated, so UVA radiation was applied to illumination.

MATERIALS AND METHODS

L-arginine diprotoporphyrinate - $PP(Arg)_2$ - was obtained from the Department of Biochemistry and Spectroscopy, Military University of Technology, Warsaw, Poland. This substance was dissolved in water to obtain 1.0; 2.0; 3.0 and 4.0 μM solutions and irradiated at 37°C, during 20 min using Emita VP-60 UVA-lamp (Łódź, Poland), with λ = 365 nm, and energy dose rate 8.3 $mW \times cm^{-2}$, measured by "E_e – meter" radiometer, made in the Department of Light Technology, University of Technology, Poznań, Poland. For study of 5-MOP effect, 1.0 μM $PP(Arg)_2$ were supplemented with 5-MOP (Sigma) stock solution (6.0 $mg \times l^{-1}$) to a 5-MOP final concentration of 210 $\mu g \times l^{-1}$, usually applied in PUVA therapy (Fossel *et al.* 1991). The obtained reaction mixtures were exposed to UVA radiation, as described above.

Neutrophils were isolated by one-step Gradisol G (Ficoll) gradient centrifugation, from heparinized blood collected from 10 healthy donors in Poznań Blood-Donation Centre. After hemolization of remaining erythrocytes with 0.84% ammonium chloride solution (pH = 7.4), the obtained cells were washed twice and suspended in phosphate-buffered saline — PBS (Biomed, Poland).

To prepare PMA stock solution, 1.0 mg of phorbol 12-myristate 13-acetate (Sigma) was dissolved in 0.1ml of dimethyl sulfoxide - DMSO (Sigma) and diluted by PBS (Biomed, Poland) to a final concentration 0.1 $mg \times l^{-1}$. Serum opsonized zymosan (OZ) was prepared according to Łabędzka *et al.* (Łabędzka, Gulyas, Schmidt & Gercken 1989). Zymosan A (Sigma) was mixed with 0.9% NaCl (POCh, Poland) solution to obtain 5.0 $g \times l^{-1}$ concentration and incubated at 100°C during 30 min. Then, zymosan was resuspensed in inactivated calf serum in proportion 1:4. This suspension was incubated at 37°C during 30 min and subsequently centrifugated to obtain zymosan precipitate which was rinsed with 0.9% NaCl (POCh, Poland) and resuspensed in PBS (Biomed, Poland) to a final concentration of 10 $g \times l^{-1}$.

For the evaluation of respiratory burst of neutrophils *in vitro*, luminol-enhanced chemiluminescence according to Allen with minor own modifications (Allen 1986) was applied. 1.77 mg of luminol (Sigma) was dissolved in 1-2 drops of 0.1 M NaOH and supplemented with PBS (Biomed, Poland) to a final volume of 1.0 ml, obtaining 0.01 M stock solution. To prepare examined samples the neutrophil supernatant contained 5×10^5 cells were supplemented with 0.5 ml of intact or UVA-exposed solutions of studied photosensitizers, with 0.015 ml of luminol stock solution, with 0.02 ml of PMA or 0.1 ml of OZ stock solutions (in the case of stimulated cells) and with PBS (Biomed,

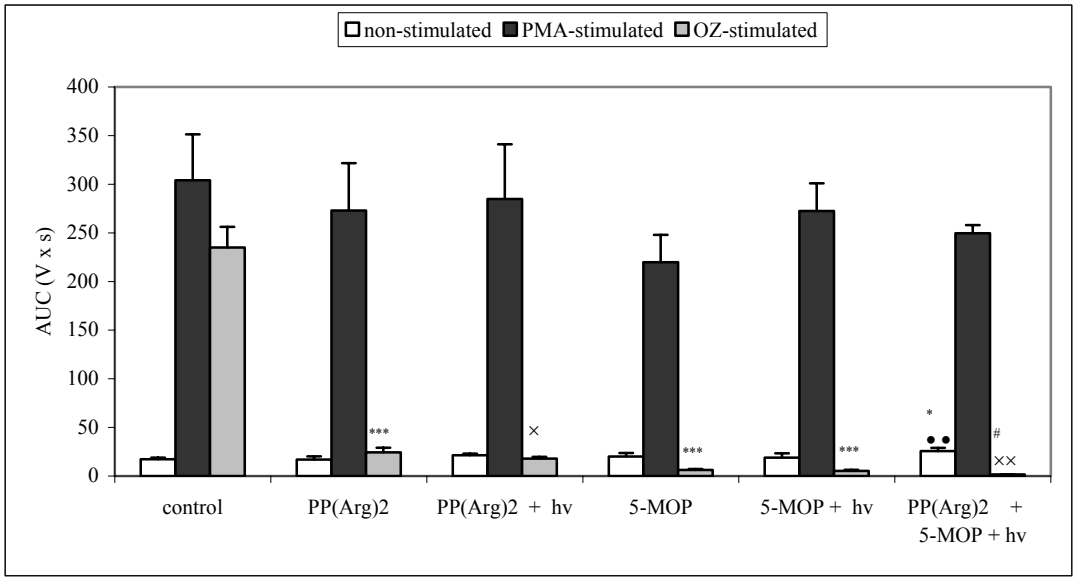

Fig. 1. Effect of non-irradiated and UVA-irradiated 1.0 μmol×l^{-1} diarginineprotoporphyrin ($PP(Arg)_2$) alone and in combination with 210 μg×l^{-1} 5-methoxypsoralen (5-MOP) towards neutrophils non-stimulated and stimulated with phorbol 12-myristate 13-acetate (PMA) and with opsonized zymosan (OZ). (Each bar represents the mean ± s.e.m. of 10 experiments performed in duplicate. *P<0.05 *vs.* control probe, t-Student's test for paired data; ***P<0.001 *vs.* control probe, two-tailed t-Student's test for paired data; $^{\#}$P<0.05 *vs.* 5-MOP, two-tailed t-Student's test for unpaired data; $^{\times}$P<0.05 *vs.* 5-MOP + hν, two-tailed t-Student's test for unpaired data; $^{\times\times}$P<0.01 *vs.* 5-MOP + hν, two-tailed t-Student's test for unpaired data; •• P<0.01 *vs.* $PP(AA)_2Arg_2$, two-tailed t-Student's test for unpaired data).

Poland) to a final volume of 1.0 ml*. The control samples did not contain photosensitizers.

The 1250 Luminometer (BioOrbit, Finland) was used for the measurement of chemiluminescence intensity. The investigations were carried out at 37ºC. Chemiluminescence was recorded for 60 minutes and the data integrated. Each measurement was expressed in V×s as an area under curve (AUC) of chemiluminescence intensity *vs.* time (Baj, Kantorski, Kowalski, Kośmider, Tchórzewski, Pawlicki & Ciećwierz 1994).

The values were expressed as mean ± standard error of mean (s. e. m.). The alpha-error probability (P) of two-tailed Student's t-test for paired and unpaired data was applied for a statistical analysis of the results of the experiments. P-value of less than 0.05 was considered to be statistically significant. The Instat (Statgraph) computer program was used to perform the statistical analysis.

* The final concentrations of $PP(Arg)_2$ were 0.5; 1.0; 1.5 and 2.0 μM and the final concentration of 5-MOP was 105 μg/l

RESULTS AND DISCUSSION

The investigation of the $PP(Arg)_2$ influence at 0.5-2.0 μM/l concentration on respiratory burst of non-stimulated neutrophils revealed no significant changes of respiratory burst intensity in samples of non-stimulated neutrophils treated with both non-irradiated and UVA-illuminated $PP(Arg)_2$ (not shown). Thus, the smallest concentration of $PP(Arg)_2$ (0.5 μM/l) was applied in further study to minimize the possibility of interactions between sensitizer compounds and other components of samples.

In the case of neutrophils stimulated with PMA in presence of non-irradiated 0.5 μM/l $PP(Arg)_2$, a decrease of AUC (272.89±48.72 V×s) comparing to control sample (304.12±47.10 V×s), was observed (Fig. 1). It may suggest, that $PP(Arg)_2$ showed antioxidative properties similarly to PPIX (Williams, Krootjes, van Steveninck & van der Zee 1994; Afonso, Vancore & Battle 1999; Cuzzocrea, Constantino, Mazzon, de Sarro & Caputti 1991). However, a statistical analysis did not show a significant difference between these two values of AUC ($P > 0.05$). In contrast, in the case of neutrophils stimulated with OZ, non-irradiated $PP(Arg)_2$ caused a stastistically significant ($P < 0.01$) decrease of the AUC value (24.37±4.69

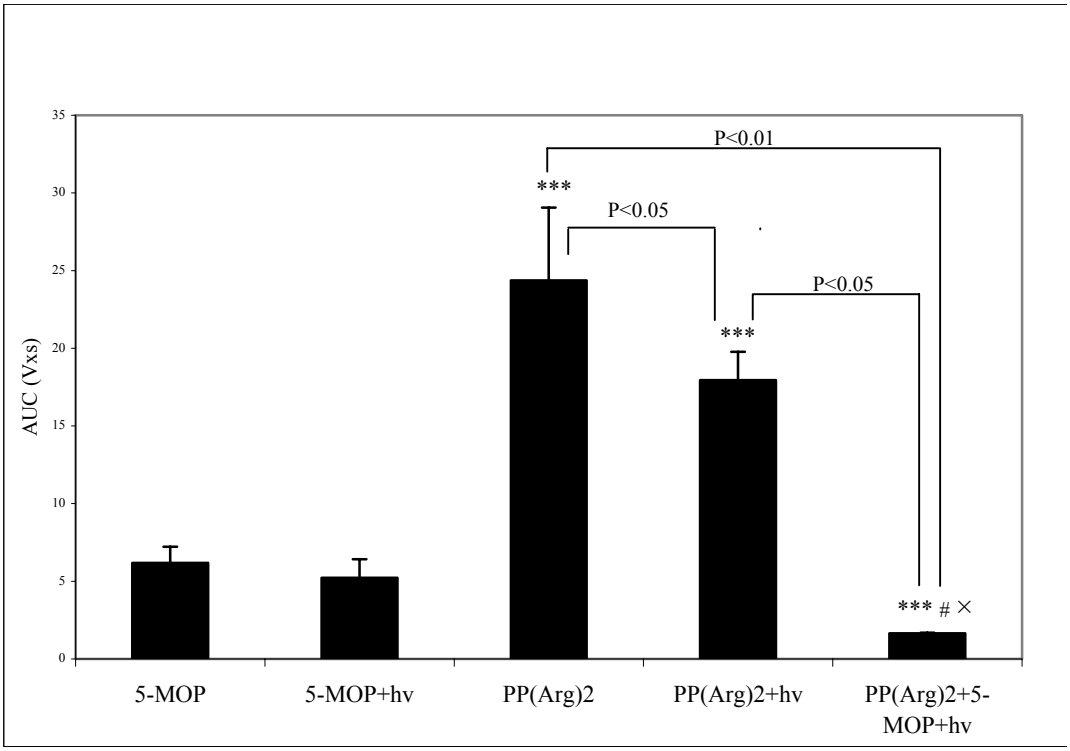

Fig. 2 Effect of 0.5 μM/l of UVA-irradiated diargininеprotoporfyrin ($PP(Arg)_2$) in combination with 105 μg/l 5-methoxypsoralen (5-MOP) on respiratory burst of OZ-stimulated neutrophils *in vitro* (Each bar represents the mean ± s.e.m. of 10 experiments performed in duplicate. # $P<0.05$ *vs.* 5-MOP, two-tailed t-Student's test for unpaired data; × $P<0.05$ *vs.* 5-MOP+hv two-tailed t-Student's test for unpaired data. P-value presented in the Figure were calculated using two-tailed t-Student's test for paired data).

V×s) in comparison to a control sample (234.89±21.34 V×s). On the other hand, no significant change of neutrophil chemiluminescence was observed under the conditions of PMA-stimulation. This suggests that $PP(Arg)_2$ inhibits epidermal growth factor-receptor tyrosine kinase, whose activity plays an important role in mechanism of OZ stimulation of leukocyte respiratory burst, while stimulatory effect of PMA is predominantly connected with release of protein kinase C (Hazan, Dana, Granot & Levy 1997). In these conditions, a decrease of respiratory burst observed in the case of OZ-stimulated neutrophils treated with $PP(Arg)_2$ might be caused by induction of superoxide dismutase (SOD), similarly to the analogical effect of PPIX and other porphyrin derivatives (Afonso *et al.* 1999; Cuzzocrea *et al.* 1999). Thus, activation of SOD by $PP(Arg)_2$ may strongly inhibit the activity of epidermal growth factor-receptor (EGF-receptor) decreasing at the same time luminol-dependent chemiluminescence induced by OZ(Ushijima, Totsune, Nishida & Nakano 1997). On the other hand, $PP(Arg)_2$ as protoporphyrin derivative could also decrease the affinity of OZ to its specific transmembrane receptors (Galon, Gauchat, Mazieres, Spagnolli, Storkus, Lotze, Bonnefoy, Fridmann & Sautes 1996; Krutmann, Athar, Mendel, Khan, Guyre, Mukhtar & Elmets 1989) thus inhibiting its stimulatory activity. However this process is connected with generation of superoxide radicals (Krutmann *et al.* 1989), while after illumination by UVA, $PP(Arg)_2$ did not also reveal a significant influence on the level of superoxide radical anion released by neutrophils stimulated with PMA, whereas in the case of OZ-stimulation, it caused a small anti-oxidative effect, statistically significant *vs.* the effect of non-irradiated $PP(Arg)_2$. This enhancement of the inhibitory effect after UVA-irradiation was similar to results of Gal *et al.* (1997) who have shown that photosensitizing porphyrins after illumination with red light significantly decrease the concentration of $O_2^{\cdot-}$ released by OZ-stimulated leukocytes, probably due to an interaction between excited triplet state of the sensitizer and doublet state of superoxide radical anion (Gal *et al.* 1997). Apart from this, UVA-illuminated $PP(Arg)_2$ undergoes transformation to photodynamic active products (Gudgin *et al.* 1995; LiWei *et al.* 2001). Thus, it could not exclude the effect

of these phototransformation products on neutrophil respiratory burst. This supposition warrant later research.

In the case of combination of 0.5 μM/l $PP(Arg)_2$ and 105 μg/l 5-MOP, a statistically significant prooxidative effect, both *vs*. control sample and non-irradiated $PP(Arg)_2$, was observed (Fig. 2). This phenomenon could be explained due to oxidation of 5-MOP by $PP(Arg)_2$ -sensitized singlet oxygen, leading to formation of H_2O_2, which reacts with luminol increasing the intensity of chemiluminescence (Marley, Larson & Davenport 1995). On the other hand, synergistic action of $PP(Arg)_2$ and 5-MOP on OZ-stimulated cells resulted in an antioxidative effect which was approximately the sum of each sensitizer action (Fig. 3). Thus, the observed phenomenon may be a the result of addition of the photodynamic effect of $PP(Arg)_2$ and the light-independent antioxidative effect of 5-methoxypsoralen. Contrary to this, no parallel antioxidative effect was observed in neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA), suggesting that neither $PP(Arg)_2$ (or its photoproducts) nor 5-MOP trigger the release of protein kinase C, which is prevalently connected with the stimulatory effect of PMA (Baj *et al.* 1994).

The obtained results do not enable to provide efficacy of $PP(Arg)_2$ in PDT therapy of cancer. To approach this question, a research using carcinoma cells would be necessary. However, this study suggest that $PP(Arg)_2$ (and probably its photoproducts) could reveal inhibitory effect towards EGF-receptor similarly to some phtalocyanine derivatives, applied in the therapy of such type of cancer as neck and head cancer, as well as lung carcinoma (Ahmad, Kalka & Mukhtar 2001). An interesting synergistic inhibitory effect of $PP(Arg)_2$ and 5-MOP towards OZ-stimulated respiratory burst of neutrophils was also demonstrated.

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