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Analysis of photoreactivity and phototoxicity of riboflavin's analogue 3MeTARF

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A B S T R A C T

Recent studies focus on usage of blue light of λ = 450 nm in combination with photosensitizers to treat surface skin disorders, including cancers. In search of convenient therapeutic factor we studied riboflavin analogue 3-methyl-tetraacetylriboflavin (3MeTARF) as potential sensitizer. Riboflavin (Rfl) itself, non -toxic in the darkness, upon absorption of UVA and blue light, may act as photosensitizer. However, Rfl efficiency is limited due to its susceptibility to photodecomposition. Riboflavin's acetylated analogue, 3MeTARF, bears substituents in ribose chain, which inhibit intramolecular processes leading to degradation. Upon excitation, this compound, reveals higher photochemical resistance, remaining a good singlet oxygen generator. Thus, being more stable as the sensitizer, might be much more efficient in photodynamic processes. The objective of undertaken study was to elucidate mechanisms of 3MeTARF photoreactivity under the irradiation with blue light in comparison to its mater compound, riboflavin. We approached this goal by using spectroscopic methods, like direct singlet oxygen phosphorescence detection at 1270 nm, EPR spin trapping and oximetry. Additionally, we tested both riboflavin and 3MeTARF phototoxicity against melanoma cells (WM115) and we studied mechanism of photodynamic cell death, as well. Moreover, 3MeTARF induces apoptosis in melanoma cells at ten times lower concentration than riboflavin itself. Our studies confirmed that 3MeTARF remains stable upon blue light activation and is more efficient photosensitizer than Rfl.

1. Introduction

Riboflavin analogues are involved in many biological processes, such as metabolic processes occurring in living cells [1], reactions occurring in cell membrane, mitochondria and cell plasma [2]. Study of flavins properties, especially in their excited states, is related to their significant contribution to the process of blue light photoreception [1]. In therapeutic aspects special interest may arouse their antitumor activity and potential role in therapy of many other diseases [3] and cited therein. Flavins were indicated as the factor in pathogene destruction and inactivation of many types of viruses and bacteria [4,5].

Their molecules are chromophores active in redox processes involved in a huge number of biotransformations. In flavoproteins, where FMN and FAD play the role of coenzyme, the active group in redox processes is the isoalloxazine system. Therefore its spectral and photophysical properties seems to be very important for better understanding flavins function in nature [6].

These compounds undergo reaction of photodynamic oxidation, which arose much interest in both harmful and potential therapeutic aspects [7–9]. Fundamental mechanisms of photosensitized oxidation occur according to Type I, *via* radicals, and Type II, which involves singlet oxygen as the transient species. Thus, many endogenous and exogenous photosensitizers, like flavins or porphyrins, may cause phototoxic and photoallergic reactions [7]. Riboflavin average concentration in a cell is too low for its photochemical activity as photosensitizer, but its potential endogenous activity *in vivo* and *in vitro* should be considered [7,10]. Flavins can sensitized oxidation of amino acids, proteins, nucleotides, lipids, vitamins. Free radicals of sensitizer, formed in electron transfer process between DNA basis and riboflavin, can activate procarcinogens and promutagens, which cause considerable risk for living organisms, leading to cell damage, inflammations or acceleration of aging processes. Photodynamic activity could occur in tissues and organs, like eyes and skin, particularly exposed to light influence [11–17].

On the other hand, generation of singlet oxygen became one of the most important processes in aspects of potential applications in therapeutic processes [18–23], as well as in the photocatalytic processes [24,25], which require strong oxidative agent. This flavins' property is the fundamental factor in photodynamic applications.

It was shown that irradiation of tumor cells with visible light in the presence of riboflavin leads to their destruction [18]. The phototoxic activity of riboflavin increases along with increasing content of tyrosine or tryptophan in reaction medium under nitrogen atmosphere.

The presence of the emerging photoproducts additionally causes cells morphological changes, resembling apoptosis [18–20,22]. Some amino acids, such as alanine or phenylalanine, inhibit the reaction of photooxidation, probably by quenching the triplet state of riboflavin, and cysteine by quenching the singlet oxygen formed in the reaction [26].

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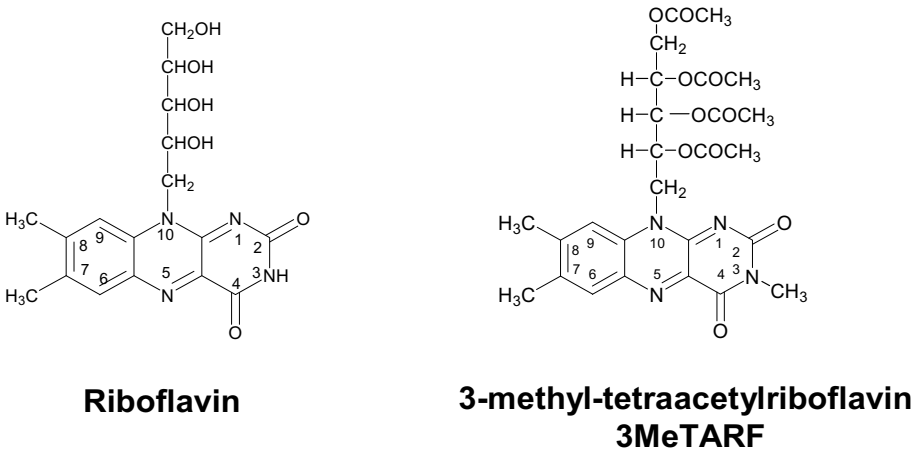


Fig. 1. Structures of riboflavin and 3-methyl-tetraacetylriboflavin (3MeTARF).

In the last decades, numerous *in vitro* research in the field of photophysics and photochemistry of flavins focuses on explaining their action in living organisms [27–29].

In the aspect of photophysical properties and photochemistry of these compounds, it was shown that under UV–Vis irradiation, flavins efficiently populate their triplet state and reacts with oxygen in the excited state [12]. Riboflavin itself can be a good sensitizer in photodegradation of many compounds, but its efficiency is limited due to its susceptibility to photodecomposition [30–35].

Recent *in vivo* and *in vitro* study [36–38], also confirmed, that riboflavin (Rfl) and tetraacetyl riboflavin (RFTA) inactive in dark, reveal good effectiveness in treating of tumor cells, like melanoma cells [38], SCC-13 cells (squamous carcinoma) and also towards *Leishmania parasites* [23]. Riboflavin is also proposed as a supportive treatment to reduce risk of hematogenous metastasis [36]. It was shown that RFTA more efficiently inhibits cell proliferation than riboflavin [37].

In the case of 3-methyl-tetraacetylriboflavin (3MeTARF), see Fig. 1, the presence of acetyl substituents in ribose chain inhibits intramolecular photoreduction [30,35,39], which cause higher photostability of the sensitizer [31]. Thus, 3MeTARF reveals higher photochemical resistance, remaining a good singlet oxygen generator [35]. Previously spectral, photophysical and photochemical properties of 3-methyl-tetraacetylriboflavin analogue were studied [32]. Properties such as good singlet oxygen sensitizing and its lower polarity predestine it to the cell study, as it may enhance permeability through cell membrane in comparison to riboflavin and its better phototoxic effect is expected [23].

The main goal of this study was to elucidate mechanism of photoreactivity of 3MeTARF under blue light irradiation in comparison to riboflavin, its mater compound, using singlet oxygen phosphorescence at 1270 nm, EPR spin trapping and oximetry. Additionally, we tested its photoactivity against melanoma cells (WM115) and we studied mechanism of photodynamic cell death.

2. Materials and Methods

2.1. Direct Detection of Singlet Oxygen ($^1\Delta_g$, $^1O_2$*) Phosphorescence at 1270nm

Before measurements, samples were dissolved in methanol or PBS and DMSO mixture (99:1, *v*/v), placed in a quartz fluorescence cuvette (QA-1000; Hellma, Mullheim, Germany) and excited with 455 nm laser pulse. Excitation light was generated by an integrated nanosecond DSS Nd:YAG laser system equipped with a narrow bandwidth optical parametric oscillator (NT242-1 k-SH/SFG; Ekspla,Vilnius, Lithuania). Quantum yield of singlet oxygen ($^1O_2$*, $^1\Delta g$), generation upon excitation with 455 nm was determined by a comparative method, employing riboflavin as a standard [40,41]. In these experiments, initial intensities of singlet oxygen phosphorescence in the studied sample and in standard excited with laser pulses were measured at increasing laser energies. Absorbance of standard and studied sample were adjusted to ~0.10 at excitation wavelength 455 nm.

The near-infrared luminescence of generated singlet oxygen was measured perpendicularly to the excitation beam in a photon-counting mode using a thermoelectric cooled NIR PMT module (H10330–45; Hamamatsu, Japan) equipped with a 1100 nm cut-off filter and a dichroic narrow band filter NBP, selectable from the spectral range 1150–1355 nm (NDC Infrared Engineering Ltd., Bates Road, Maldon, Essex, UK). Data were collected using a computer-mounted PCI board multichannel scaler (NanoHarp 250; PicoQuant GmbH, Berlin, Germany). Data analysis, including first-order luminescence decay fitted by the Levenberg–Marquardt algorithm, was performed by custom-written software.

2.2. EPR Oximetry and Spin Trapping

Measurements were carried out during *in situ* irradiation of the samples, placed in the resonant cavity as previously described [42]. Blue light (445 nm, 1.8 mW/cm^2) was derived from a 10 W diode array illuminator (High Power LED Chip Royal Blue 445 nm, Shen Zhen Yong Xing Optoelectronics Co., Ltd., China). Oxygen consumption was measured by EPR oximetry using 0.1 mM mHCTPO as an oxygen-sensitive spin probe according to a method described elsewhere [43,44]. The apparatus settings were as follows: center field 337.67 mT, sweep width 0.3 mT, microwave power 1.06 mW, modulation amplitude 0.006 mT, time constant 40.960 ms and sweep time 5.243 s. EPR spin trapping measurements were performed using DMPO as a spin trap employing the same light source as those described above. Samples typically contained 200 μM riboflavin or 3MeTARF. Time-dependent photo-accumulation of the DMPO-OOH spin adducts was measured in 90% DMSO at DMPO concentration 0.1 M. Light-induced accumulation of DMPO-OH and DMPO-N_3 was measured in water in the presence of 0.01 M DMPO. EPR spin-trapping measurements were performed at the following parameters: microwave power 10.6 mW, modulation amplitude 0.05 mT, scan width 8 mT, and scan time 84 s. The values of hyperfine splitting constants for the DMPO-OOH spin adduct have been taken from [45] for DMPO-OH from [45,46] and for DMPO-N3 from [47].

2.3. Cell Culture and LED Irradiation

WM115 human, skin melanoma cell line was purchased from ATCC. WM115 cells were cultured in in RPMI1640 supplemented with 10%

FBS (Lonza) and 1% of antibiotics. The cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2 and passaged at c.a. 70–80% of confluence. 1×10^5 cells were seeded on 24 well plate 24 h before the treatment. Thereafter, the medium was removed and the cells were incubated for 15 min in the darkness with riboflavin (0–50 μM) or 3-MetTARF (0–10 μM). Both flavins were dissolved in DMSO. The concentration of DMSO 0.5% in PBS, supplemented with 0.01% $MgCl_2$ and 0.01% $CaCl_2$. The plates were illuminated at room temperature *via* a lid, using LED lamps emitting 438 nm blue light (PXM sp.k. Zupnik M., Poland) for 10–15 min. The integrated irradiance of the lamp measured using a LI-250 A radiometer equipped with probe PY (LI-COR) was 50 W/m^2. Control cells were kept in the dark under similar conditions.

2.4. DHR 123

The detection of intracellular production of ROS was performed using dihydrorhodamine 123 (DHR123; Sigma Aldrich) directly after irradiation, as described before [48].

2.5. MTT

For MTT cell viability assay, Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma) was added for 60 min at a final concentration of 500 ng/ml. The medium was removed by suction and MTT crystals were dissolved in DMSO: EtOH (1:1). The absorbance was measured at 560 nm in a plate reader (Tecan Genios, Männedorf, Switzerland) [48].

2.6. AnnexinV-FITC/7AAD

Annexin V and 7-amino-actinomycin D (7AAD) staining was performed as described previously [48]. The cells were collected by trypsinization 24 h after the irradiation, washed with Hepes buffer and incubated with AnnexinV-FITC Apoptosis Detection Kit (Bender MedSystem, Austria) in the dark for 10 min. Thereafter, the cells were washed with Hepes buffer, resuspended and stained with 5 μl 7-AAD for additional 5 min. Then, 10-000 cells were collected by FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest (BD Biosciences) software. The measurement was carried out using 488 nm excitation and a 510–570 nm band-pass emission filter for the detection of fluorescein isothyocyanate (FITC) and a 650 nm long-pass emission filter for 7-AAD detection.

2.7. Statistics

All results are the means of at least three independent experiments ± standard deviation (SD). The data was analyzed using *Student's t-test* in Excel (Microsoft). Statistical significance was accepted at the level of $P < .05$.

3. Results

Both riboflavin and 3MeTARF in PBS/DMSO (95:5, *v*/v), mixture used in cells irradiation, reveal similar absorption spectra and both flavins absorb in UV/Vis range with maxima at about 372–374 nm and

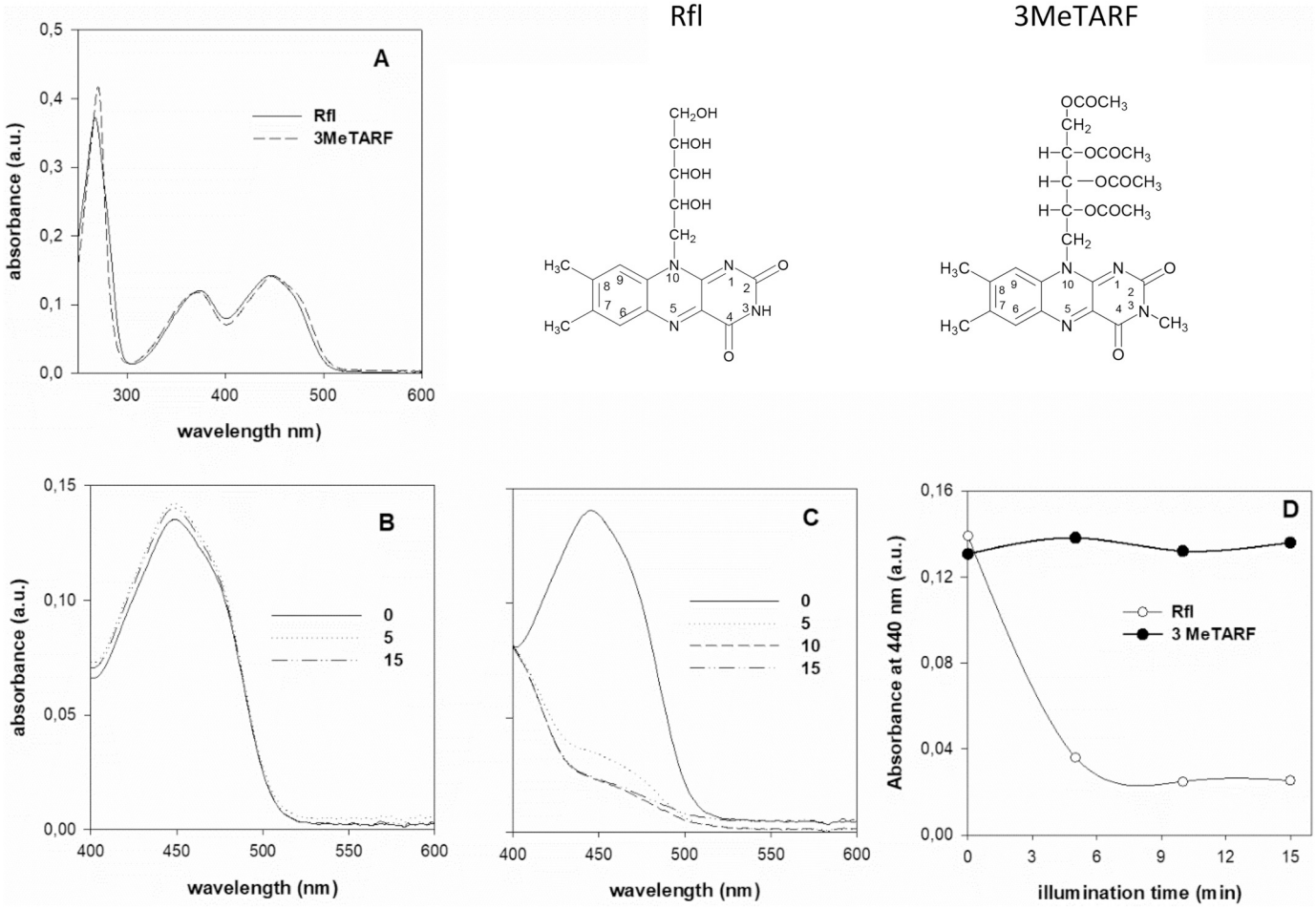


Fig. 2. Absorption spectra of riboflavin and 3MeTARF (10 μM; PBS:DMSO; 95:5, *v*/v) (A) and after illumination of 3MeTARF (B) and riboflavin (C) with 438 nm blue LED (50 W/m^2) for 5, 10 and 15 min. Rate of photodegradation of studied flavins (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

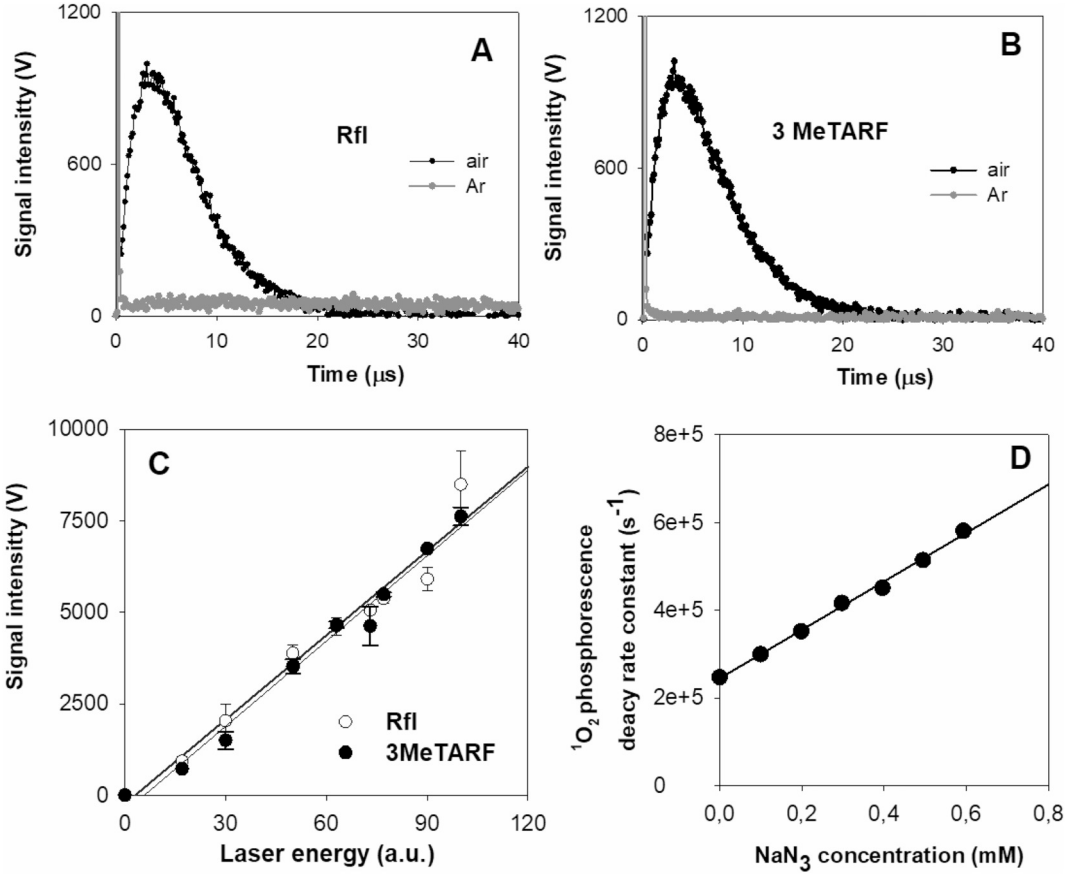


Fig. 3. Singlet oxygen ($^1O_2$, $^1\Delta g$) phosphorescence decay detected at 1270 nm after laser excitation of Riboflavin (A) and 3MeTARF (B) in PBS:DMSO (99:1,*v*/v) solution with 455 nm. Flavins solutions were equilibrated with air (black) or saturated with argon (grey). Dependence of phosphorescence intensity of singlet oxygen generated by Riboflavin and 3MeTARF on excitation energy (C). Impact of efficient singlet oxygen quencher – sodium azide on singlet oxygen decay rate constant. Singlet oxygen generated by 3MeTARF (D).

Table 1
Quantum yields (ΦΔ) and lifetimes (τΔ) of singlet oxygen ($^1O_2$, $^1\Delta g$) generation by 3MeTARF in methanol and PBS:DMSO (99:1, v/v) homogenous solutions upon excitation with 455 nm laser pulse. Riboflavin was used as a standard of well-known quantum yield of $^1O_2$ generation.

Compound	Methanol (CH_3OD)		PBS:DMSO (99:1, v/v)	
	ΦΔ	τΔ (μs)	ΦΔ	τΔ (μs)
Riboflavin	0.48 [40]	10.05 ± 0.12	0.49 [53]	3.86 ± 0.04
3MeTARF	0.46 ± 0.05	10.03 ± 0.04	0.49 ± 0.05	3.88 ± 0.06

446–450 nm, respectively (Fig. 2A). Exposure of the compounds to blue light (λ = 438 nm), as expected on the basis of previous study [32,49,50], leads to photodegradation of Rfl in contrast to 3MeTARF (Fig. 2B–D), which remains quite stable.

3.1. Photogeneration of Singlet Oxygen

Both riboflavin and 3MeTARF generate singlet oxygen upon irradiation with blue light with similar efficiency. Decays of characteristic $^1O_2$ phosphorescence generated by 3MeTARF and riboflavin in PBS:DMSO (99:1, *v*/v) solution equilibrated with air upon excitation with laser pulse are presented in Fig. 3A and B. The observed phosphorescence of $^1O_2$, generated by riboflavin and 3MeTARF, decayed with time constant expected for singlet oxygen lifetime in water *i.e.* 3.86 (±0.04) μs and 3.88 (±0.06) μs, respectively [51]. Lifetime of singlet oxygen generated by riboflavin and 3MeTARF in methanol was longer and amounted 10.05(±0.12) μs and 10.03 (±0.04) μs respectively, in agreement with previously reported data (Table 1) [52]. After saturation of studied flavins solutions with argon, singlet oxygen luminescence was not observed (Fig. 3A, B). Using riboflavin as singlet oxygen generation standard, quantum yield of $^1O_2$ ($^1\Delta_g$) generation by 3MeTARF was determined in methanol and PBS containing 1% (*v*/v) of DMSO. While quantum yield of $^1O_2$ ($^1\Delta_g$) generation by 3MeTARF determined in methanol yielded 0.46 (±0.05) and was slightly lower than that of riboflavin (0.48) [40], in PBS:DMSO solution it reached 0.49 and was the same as that of riboflavin (0.49) [53] (Table 1, Fig. 3C).

In order to show involvement of $^1O_2$ in the process, the azide ion was used as a strong physical quencher of singlet molecular oxygen ($^1O_2$). For this purpose rate constant of sodium azide (NaN_3) interaction with singlet oxygen, generated by 3MeTARF in PBS:DMSO (99:1, *v*/v) solution was determined. Increasing rate constant of decay of $^1O_2$ phosphorescence observed in the presence of increasing concentration of sodium azide is presented in Fig. 3D. Appointed rate constant of $^1O_2$ quenching by NaN_3 was 5.53×10^8 M^{-1} s^{-1}, which was very close to the previously reported data [54].

3.2. Oxygen Photo-Consumption

The spin probe, mHCTPO, undergoes degradation during irradiation of riboflavin or 3MeTARF in the presence of histidine (Fig. 4A). Our earlier studies showed that error of oxygen concentration does not exceed 10% if decrease of mHCTPO concentration is not greater than 50%. That occurred after 2 min and 1.25 min of irradiation of sample containing riboflavin (Fig. 4B) and 3MeTARF, respectively (Fig. 4C). For longer irradiation time, calculated oxygen concentrations in the

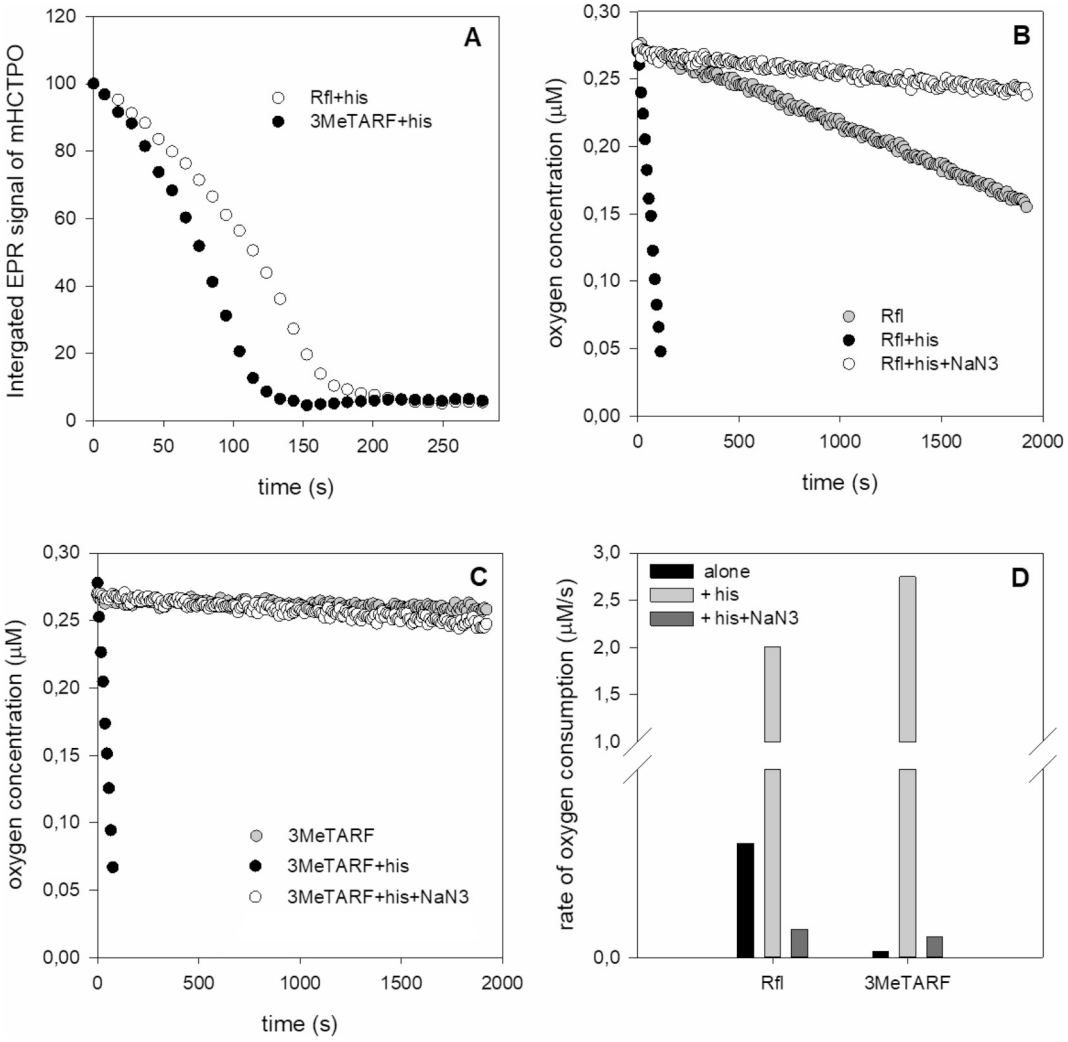


Fig. 4. The kinetics of light-induced mHCTPO degradation (A) and oxygen consumption (B,C) in the samples containing 200 μM riboflavin (B) or 200 μM 3MeTARF (C), irradiated with 445 nm light (1.8 mW/cm^2). The initial rate of oxygen consumption (D).

samples with histidine were not reliable. In the absence of histidine, oxygen consumption was observed only in the case of riboflavin (Fig. 4C,B,D). This result confirms that singlet oxygen or free radicals formed during studied flavins irradiation react with riboflavin but not with 3MeTARF. These data clarify riboflavin susceptibility to photodegradation (Fig. 2). Very fast oxygen consumption observed in the presence of histidine was almost totally inhibited by sodium azide (Fig. 4B,C,D), which indicates that singlet oxygen is the main reactive oxygen species responsible for observed oxygen consumption.

3.3. Generation of Free Radicals

In order to study the ability of Rfl and 3MeTARF to generate free radicals, samples containing the studied flavins were irradiated with light 445 nm. Illumination of flavin solution in 90% DMSO with 0.1 M DMPO caused rapid DMPO-OOH accumulation (Fig. 5A). The initial rate of this process was similar in the case of both studied flavins, which suggests that the yield of superoxide anion photogeneration is by 3MeTARF is comparable to that of riboflavin. Further irradiation caused decrease of intensity of EPR signal of DMPO-OOH spin adduct. However, observed signal loss occurs earlier in the case of 3MeTARF than for riboflavin. Destruction of this spin adduct can be caused either by excited compounds or by formed reactive oxygen species (ROS). Such result might suggest, that since 3MeTARF did not undergo photodegradation, can more efficiently destroy the adduct. If riboflavin and 3MeTARF were irradiated in water, EPR spectrum typical for DMPO-OH was registered (Fig. 5B). However, the kinetics was difficult to interpret (Fig. 5B) since DMPO-OOH rapidly converts to DMPO-OH in water and superposition of three effects is observed: DMPO-OOH conversion, DMPO-OH decay, and probably, DMPO-OH formation. In order to check whether $^{\cdot}$OH radical is really formed, we added sodium azide to the sample. Indeed, irradiation of samples with 0.025 M NaN_3 caused formation of spin adduct typical for DMPO-N_3, although contaminated with DMPO-OH. Obtained result confirms light-induced OH radical formation. In the case of 3MeTARF this process occurs faster than for Rfl, which indicates that 3MeTARF generates hydroxyl radical more efficiently than riboflavin (Fig. 5C). Longer irradiation of the sample with 3MeTARF, unlike riboflavin sample, caused gradual decay of DMPO-N_3, which indicates destruction of such spin adduct. This process can be explained similarly as in the case of DMPO-OOH disappearance (*vide supra*).

3.4. Phototoxicity of 3MeTARF vs. Riboflavin

In order to investigate the phototoxic effect we irradiated WM115 cells in the presence of Rfl or 3MeTARF following 15 min of dark incubation. While no toxic effect on the cells is observed in the presence of both flavins in darkness (see Fig. 6), upon illumination with blue light 438 nm in the presence of Rfl or 3MeTARF decreases cell viability, which was determined by MTT assay in a dose-dependent way.

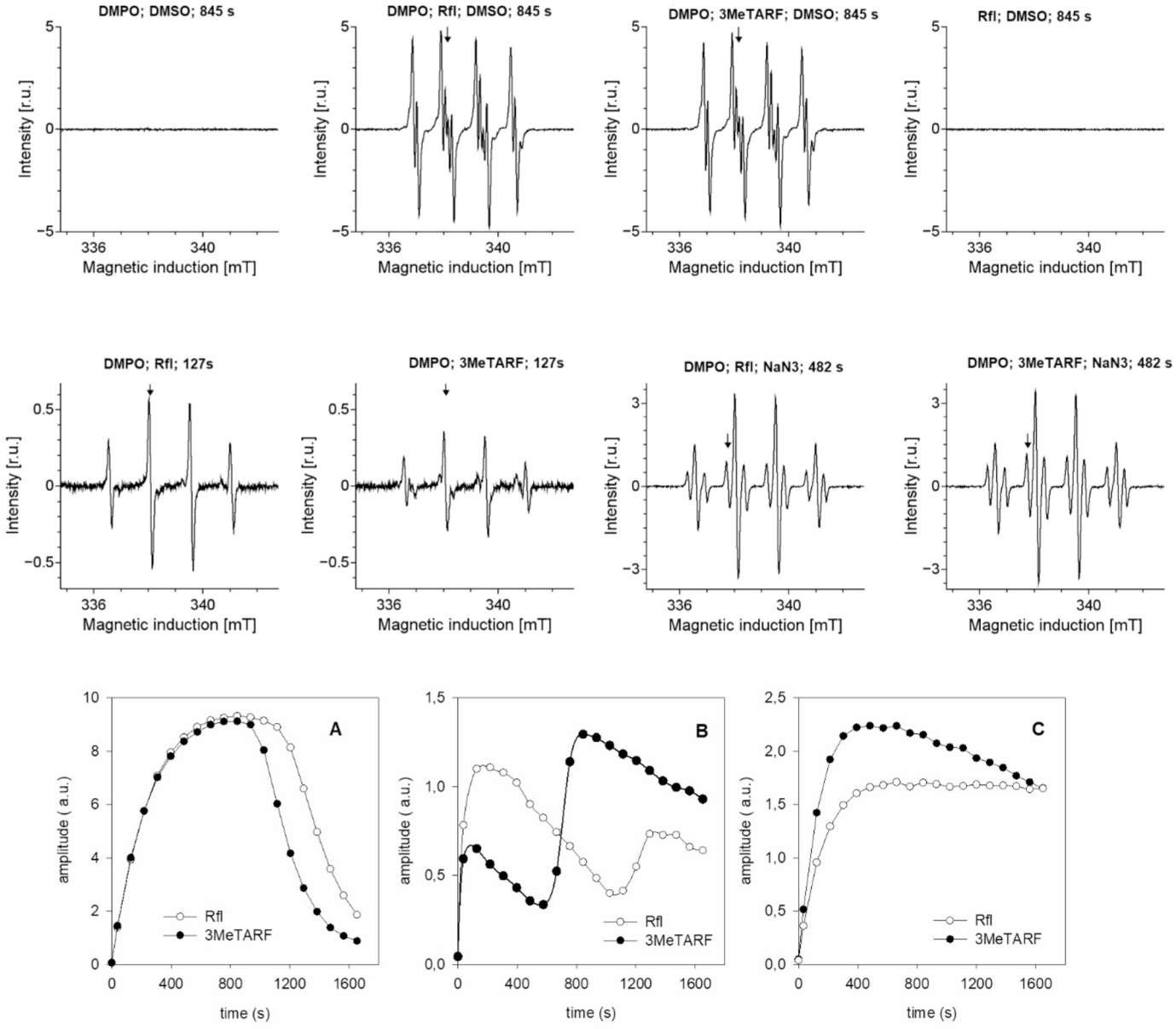


Fig. 5. Upper panel – EPR spectra of spin adducts for selected time of irradiation. Arrows indicate the line at which time was measured and signal amplitude was determined. Lower panel: Light-induced accumulation of DMPO-OOH (A), DMPO-OH (B) or DMPO-N3 (C). Samples contained 200 μM riboflavin or 3MeTARF in 90% DMSO with 0.1 M DMPO (A), water with 0.01 M DMPO (B) or water with 0.01 M DMPO and 0.025 mM NaN3 (C) were irradiated with light (1.8 mW/cm^2).

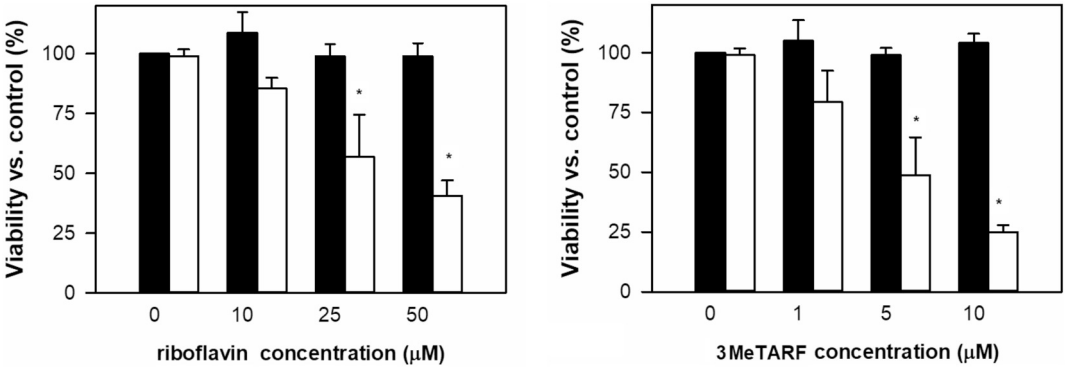
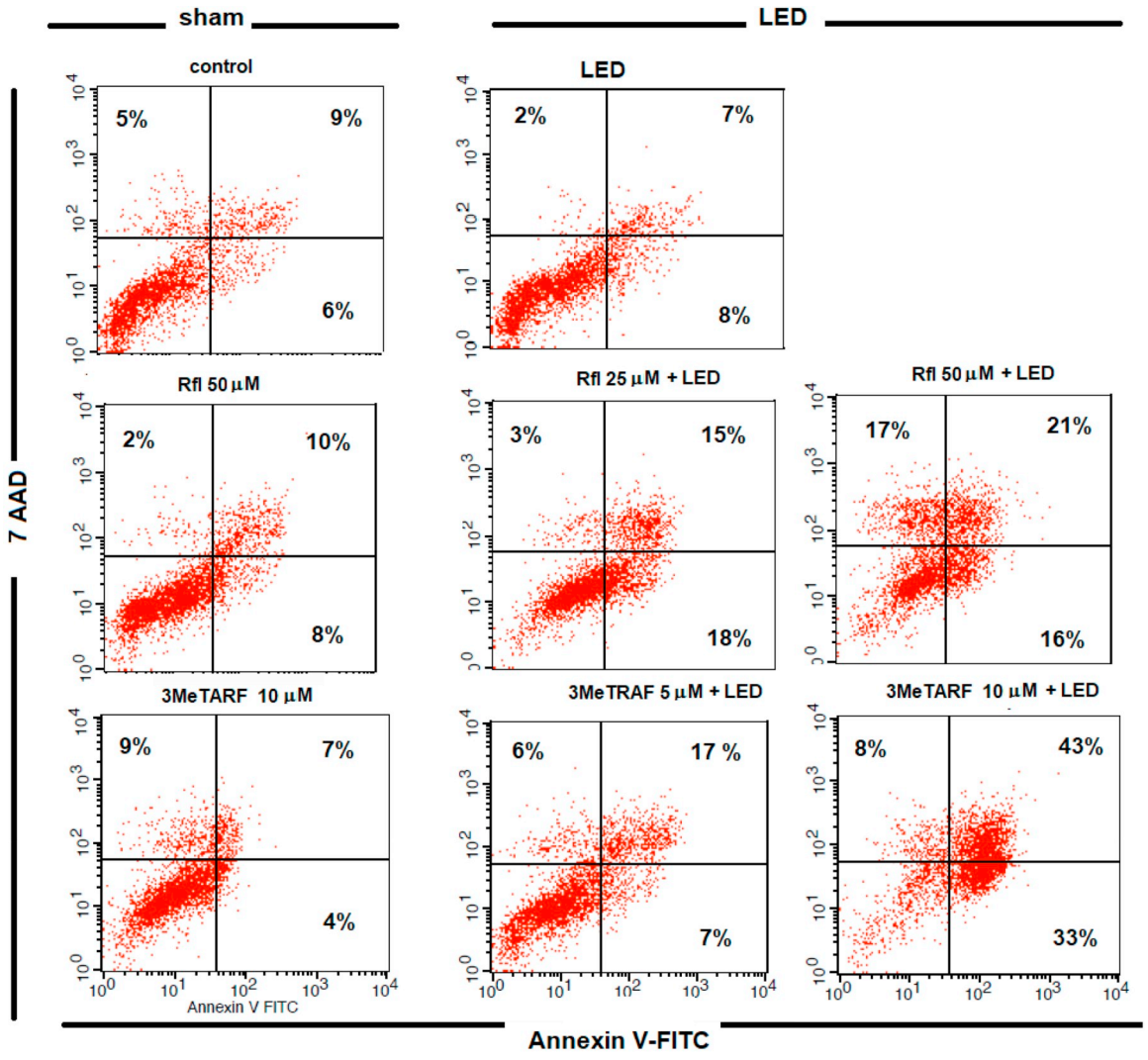


Fig. 6. Viability 24 h after the treatment WM115 cells with riboflavin (10- 50 μM) and 3MeTARF (1-10 μM) in the darkness (black) and irradiated with blue LED light (white) for 15 min (white; 50 mW/cm^2) by MTT assay. The graphs show mean ± SD from three independent experiments. For the statistics *t*-test was performed: $P < .05$, * *versus* dark control.



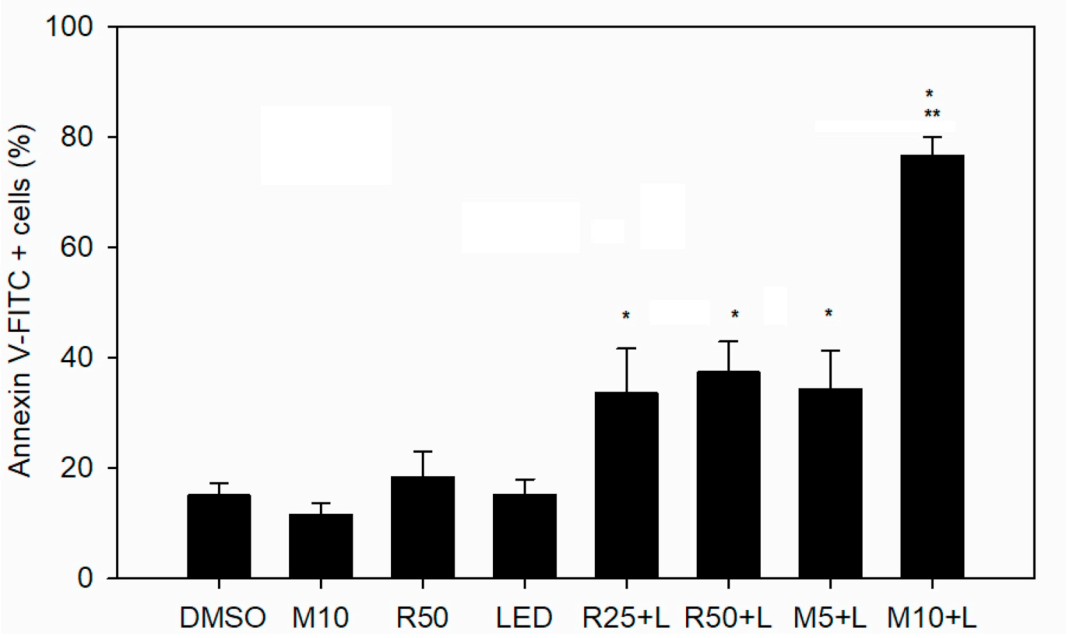


Fig. 7. Annexin V-FITC/7AAD double staining of WM115 cells irradiated with blue light (LED; L) in the presence of riboflavin (R; 25 and 50 µM) or 3MeTARF (M; 5 and 10 µM). Graph represents mean of three independent experiments ± SD. For the statistics *t*-test was performed: $P < .05$, * *versus* dark control, ** *verus* irradiated samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

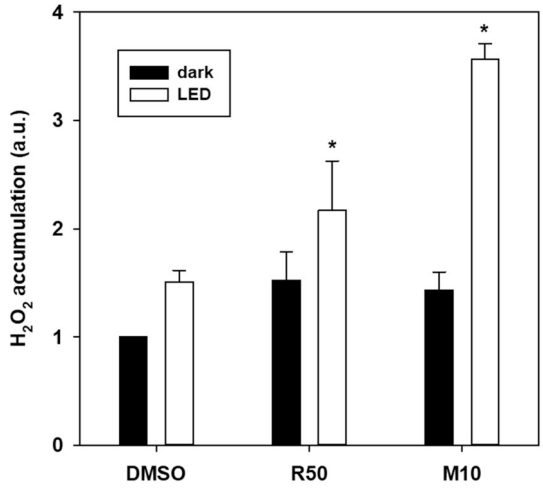


Fig. 8. The accumulation of H_2O_2 directly after blue light exposure was analyzed by DHR123 assay. WM115 cells with riboflavin (R; 50 μM) and 3MeTARF (M; 10 μM) in the darkness (black) and irradiated with blue LED light (white) for 15 min (white; 50 mW/cm^2) by MTT assay. The graphs show mean ± SD from three independent experiments. For the statistics *t*-test was performed: $P < .05$, * *versus* dark control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Moreover, 3MeTARF induces similar phototoxic effect on tested cell line at almost 5 times lower concentration, indicating that is more effective photosensitizer.

Both flavins tested upon irradiation induce cells apoptosis (Fig. 7). However, WM115 cell line turned out more resistant to Rfl than to 3MeTARF. Riboflavin at concentration of 50 μM induced about 40% of apoptosis (AnnV+ cells), while 3MeTARF: 5 μM about 34%, 10 μM - 80%, which is consistent with results obtained by MTT assay.

To determine the role of reactive oxygen specious (ROS) in cell death induced by photoactivated flavins we performed DHR 123 (dihydrorhodamine 123) assay. DHR 123 is an uncharged and non-fluorescent reactive oxygen species (ROS) indicator that can passively diffuse across membranes. In the presence of ROS generated within the cell, DHR is oxidized to fluorescent cationic rhodamine 123, which localizes in the mitochondria. Increase of rodamine 123 fluorescence intensity correlates with intracellular accumulation of H_2O_2 and other ROS which correlate with oxidative stress. We found that 3MeTARF, upon irradiation at 5 times lower concentration than riboflavin, more efficiently increase H_2O_2 accumulation in WM 115 cells (3.56 ± 0.14 *vs.* 2.17 ± 0.45) (Fig. 8). An increase in intracellular level of ROS induced by 3MeTARF + LED correlates with more efficient apoptosis.

4. Discussion

Riboflavin and some of its derivatives are known as good singlet oxygen sensitizers [39] and regarded as potential agents in numerous processes based on photodynamic actions [4,55,56], including photodynamic therapy. In presented paper riboflavin and its tetraacetyl analogue, 3MeTARF, have been studied in the aspects of their interactions with oxygen and potential activeness towards cancer cells. For this purpose we investigated both compounds with special attention paid to singlet oxygen generation ability, oxygen consumption, generation of free radicals and phototoxicity towards cancer cells (WM115).

In the course of PDT an increase level of reactive oxygen species (ROS) is produced and their amount depends on many factors, *i.e.* the light intensity, oxygen concentration, local concentration of photosensitizer, its molar absorption coefficient, quantum yield of $^1O_2$ generation ect. [37,57].

Riboflavin is well known as very efficient endogenous sensitizer of singlet oxygen generation with quantum yields of 0.54 and 0.49 upon excitation at 355 nm [58] and 445 nm [52], respectively. Riboflavin's analogue studied in this work - 3MeTARF, excited with 445 nm laser pulse, generates singlet oxygen with similar efficiency reaching 0.46 and 0.49 in methanol and PBS:DMSO (99:1, *v*/v) solutions, respectively (Table 1.). Such high quantum yields of $^1O_2$ generation characterizes also other flavins (lumiflavin, FMN) and products of their decomposition (alloxazines) [41,59,60].

After excitation sensitizer can react with species produced in the result of their photoactivation. It has been shown that riboflavin efficiently interacts with singlet oxygen with rate constant 6.0×10^7 M^{-1} s^{-1} [40]. In addition, Huang et al. reported even higher value of riboflavin and singlet oxygen interaction 1.01×10^{10} M^{-1} s^{-1}, suggesting that electrophilic attack of singlet oxygen on riboflavin is responsible for its rapid photodegradation [49]. Later on, the same authors published slightly lower reaction rate for riboflavin and singlet oxygen: 9.66×10^8 M^{-1} s^{-1} and revealed that singlet oxygen was also involved in the photosensitized degradation of lumiflavin and lumichrome, which interact with $^1O_2$ with similar rates 8.58×10^8 and 8.21×10^8 M^{-1} s^{-1}, respectively [61]. In the case of 3MeTARF determined rate constant of interaction with singlet oxygen was low and did not exceed 1×10^5 M^{-1} s^{-1}, which suggests that 3MeTARF is not a subject of dynamic, electrophilic attack of singlet oxygen. Indeed, it seems that 3MeTARF is resistant to light-induced bleaching and did not degrade upon irradiation (Fig. 2).

On the basis of the study we declare that reactive oxygen species, formed during 3MeTARF irradiation, do not interact with its molecules and the main specie formed in this process is singlet oxygen. On the other hand, 3MeTARF generates also hydroxyl radical and even more efficiently than riboflavin.

Photochemical resistance of 3MeTARF, on one hand might be due to its different structure *vs,* riboflavin, as hydroxyl groups in ribose chain are blocked, which prevents from light-induced intramolecular processes leading to molecule decomposition [9,62,63]. Relatively small value of the rate constant in the reaction with singlet oxygen (1×10^5 M^{-1} s^{-1}) may confirm 3MeTARF resistance to photobleaching and stability upon excitation. Additionally, such structure with acetyl substituents in the ribityl chain increase hydrophobicity of the molecule, which facilitates permeability through cell membrane and raise the effectiveness towards cell killing [23].

We found that 3MeTARF increases oxidative stress more efficiently than riboflavin in WM115 melanoma cells. This is in agreement with results obtained by spin trapping methods, which showed that although both flavins similarly generate superoxide anion, 3MeTARF more efficiently produce hydroxyl radical. DHR 123 is suggested to be oxidized specifically by H_2O_2 however it was shown that other oxidative specieus can also oxidase DHR123 [64,65]. Rappole et al. showed that DHR is sensitive to ·OH· influx generated by X-ray in live cells [66].

Results obtained in cell viability test (MTT test) and by flow cytometry (Annexin V- FITC/7AAD) indicates that both riboflavin and 3MeTARF are effective sensitizers in induction of cell death towards cell line WM115. We observed that phototoxic effect in the case of 3MeTARF is 5 times more efficient when compared to riboflavin. Moreover, we observe apoptosis, which is much more desired effect from therapeutic point of view. Avoiding necrosis may limit side-effects and complications, like inflammations of surrounding tissues [37,66].

5. Conclusions

Tetracetyl riboflavin analogue (3MeTARF), similar to Rfl in its spectral properties and singlet oxygen generation abilities, reveals higher stability upon irradiation, which makes it more efficient as potential therapeutic agent, which is not without significance in the aspects of potential PDT applications. Moreover, its increased hydrophobicity may facilitate permeability through cell membrane, thus

increasing its bioavailability. Obtained results confirm good properties of 3MeTARF, riboflavin derivative, as potential sensitizer in photodynamic reactions. Effects of investigations show huge potential that lies in riboflavin and its derivatives as the available therapeutic factors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] W. Holzer, J. Shirdel, P. Zirak, A. Penzkofer, P. Hegemann, R. Deutzmann, E. Hochmuth, Photo-induce degradation of some flavins in aqueous solution, Chem. Phys. 308 (2005) 69–78.
[2] H. Grajek, G. Zurkowska, J. Kuśba, Influence of diffusion on nonradiative energy transfer between FMN molecules in aqueous solutions, J. Photochem. Photobiol. B 80 (2005) 145–155.
[3] H.I. Ali, N. Ashida, T. Nagamatsu, Antitumor studies. Part 4: Design, synthesis, antitumor activity, and molecular docking study of novel 2-substituted 2-deoxoflavin-5- oxides, 2-deoxoalloxazine-5-oxides, and their 5-deaza analogs, Bioorg. Med. Chem. 16 (2008) 922–940.
[4] F. Corbin, Pathogen inactivation of blood components: current status and introduction of an approach using riboflavin as a photosensitizer, Int. J. Hematol. 76 (2002) 253–257.
[5] H.L. Reddy, A.D. Dayan, J. Cavagnaro, S. Gad, J. Li, R.P. Goodrich, Toxicity testing of a novel riboflavin-based technology for pathogen reduction and white blood cell inactivation, Transfus. Med. Rev. 22 (2008) 133–153.
[6] S. Frago, M. Medina, J.I. Martinez, Theoretical Analysis of the Changes Introduced in the Electron Spin Density Distribution of the Natoral Flavin Semiquinone Isoalloxazine Ring Upon Modification of the Isoalloxazine Ring, Rudolf Weber, Berlin, (2002), pp. 175–176.
[7] J. Iqbal, A. Husain, A. Gupta, Photooxidation of acyclovir with thermally generated triplet excited ketones. A comparison with Type I and II photosensitizers, Chem. Pharm. Bull. 54 (2006) 519–521.
[8] C.S. Foote, Definition of type I and type II photosensitized oxidation, Photochem. Photobiol. 54 (1991) 659.
[9] M. Insińska-Rak, M. Sikorski, Riboflavin interactions with oxygen-A survey from the photochemical perspective, Chem. Eur. J. 20 (2014) 15280–15291.
[10] S.O. McDonnell, M.J. Hall, L.T. Allen, A. Byrne, W.M. Gallagher, D.F. O'Shea, Supramolecular photonic therapeutic agents, J. Am. Chem. Soc. Comm. 127 (2005) 16360–16361.
[11] C.Y. Lu, W.F. Wang, W.Z. Lin, Z.H. Han, S.D. Yao, N.Y. Lin, Generation and photosensitization properties of the oxidized radical of riboflavin a laser flash photolysis study, J. Photochem. Photobiol. B 52 (1999) 111–116.
[12] C.Y. Lu, W.Z. Lin, W.F. Wang, Z.H. Han, Z.D. Zheng, S.D. Yao, Kinetic observation of rapid electron transfer between pyrimidine electron adducts and sensitizers of riboflavin, flavin adenine dinucleotide (FAD) and chloranil: a pulse radiolysis study, Radiat. Phys. Chem. 59 (2000) 61–66.
[13] C.Y. Lu, Z.H. Han, G.S. Liu, X.C. Cai, Y.L. Chen, S. Yao, Photophysical and photochemical processes of riboflavin (vitamin B-2) by means of the transient absorption spectra in aqueous solution, Sci. China Ser. B 44 (2001) 39–48.
[14] J.M. King, D.B. Min, Riboflavin-photosensitized singlet oxygen oxidation product of vitamin D-2, J. Am. Oil Chem. Soc. 79 (2002) 983–987.
[15] J.M. King, D.B. Min, Riboflavin photosensitized singlet oxygen oxidation of vitamin D, J. Food Sci. 63 (1998) 31–34.
[16] J.Y. Liang, J.M. Yuann, C.W. Cheng, H.L. Jian, C.C. Lin, L.Y. Chen, Blue light induced free radicals from riboflavin on *E. coli* DNA damage, J. Photochem. Photobiol. B 119 (2013) 60–64.
[17] D.R. Cardoso, S.H. Libardi, L.H. Skibsted, Riboflavin as a photosensitizer. Effects on human health and food quality, Food Funct. 3 (2012) 487–502.
[18] A.M. Edwards, E. Silva, Effect of visible light on selected enzymes, vitamins and amino acids, J. Photochem. Photobiol. B 63 (2001) 126–131.
[19] A.M. Edwards, C. Bueno, A. Saldano, E. Silva, K. Kassab, L. Polo, G. Jori, Photochemical and pharmacokinetic properties of selected flavins, J. Photochem. Photobiol. B 48 (1999) 36–41.
[20] A.M. Edwards, E. Silva, B. JOFRE, M.I. Becker, A.E. DeIoannes, Visible-light effects on tumoral cells in a culture-medium enriched with tryptophan and riboflavin, J. Photochem. Photobiol. B 24 (1994) 179–186.
[21] E. Silva, A.M. Edwards, D. Pacheco, Visible light-induced photooxidation of glucose sensitized by riboflavin, J. Nutr. Biochem. 10 (1999) 181–185.
[22] E. Silva, S. Furst, A.M. Edwards, M.I. Becker, A.E. DeIoannes, Visible light anaerobic photoconversion of tyrosine sensitized by riboflavin. Cytotoxicity on mouse tumoral cells, Photochem. Photobiol. 62 (1995) 1041–1045.
[23] A.V. Silva, A. Lopez-Sanchez, H.C. Junqueira, L. Rivas, M.S. Baptista, G. Orellana, Riboflavin derivatives for enhanced photodynamic activity against Leishmania parasites, Tetrahedron 71 (2015) 457–462.
[24] A.A. Linden, L. Kruger, J.E. Backvall, Highly selective sulfoxidation of allylic and vinylic sulfides by hydrogen peroxide using a flavin as catalyst, J. Organomet. Chem. 68 (2003) 5890–5896.
[25] A.A. Linden, N. Hermanns, S. Ott, L. Kruger, J.E. Backvall, Preparation and redox properties of N,N,N-1,3,5-trialkylated flavin derivatives and their activity as redox catalysts, Chem. Eur. J. 11 (2004) 112–119.
[26] M. Sun, T.A. Moore, P.S. Song, Molecular luminescence studies of flavins. I. The excited states of flavins, J. Am. Chem. Soc. 94 (1972) 1730–1740.
[27] A. Penzkofer, Photoluminescence behavior of riboflavin and lumiflavin in liquid solutions and solid films, Chem. Phys. 400 (2012) 142–153.
[28] W. Holzer, A. Penzkofer, M. Fuhrmann, P. Hegemann, Spectroscopic characterization of flavin mononucleotide bound to the LOV1 domain of Phot1 from Chlamydomonas reinhardtii, Photochem. Photobiol. 75 (2002) 479–487.
[29] C.B. Martin, M.L. Tsao, C.M. Hadad, M.S. Platz, The reaction of triplet flavin with indole. A study of the cascade of reactive intermediates using density functional theory and time resolved infrared spectroscopy, J. Am. Chem. Soc. 124 (2002) 7226–7234.
[30] R.A. Larson, P.L. Stackhouse, T.L. Crowley, Riboflavin tetraacetate: a potential photosensitizing agent for the treatment of contaminated waters, Environ. Sci. Technol. 26 (1992) 1792–1798.
[31] M. Insińska-Rak, A. Golczak, M. Sikorski, Photochemistry of riboflavin derivatives in methanolic solutions, J. Phys. Chem. A 116 (2012) 1199–1207.
[32] M. Insinska-Rak, E. Sikorska, J.L. Bourdelande, I.V. Khmelinskii, W. Prukala, K. Dobek, J. Karolczak, I.F. Machado, L.F.V. Ferreira, E. Dulewicz, A. Komasa, D.R. Worrall, M. Kubicki, M. Sikorski, New photochemically stable riboflavin analogue - 3- methyl-riboflavin tetraacetate, J. Photochem. Photobiol. A 186 (2007) 14–23.
[33] I. Ahmad, Q. Fasiliullah, F.H.M. Vaid, A study of simultaneous photolysis and photoaddition reactions of riboflavin in aqueous solution, J. Photochem. Photobiol. B 75 (2004) 13–20.
[34] I. Ahmad, S. Ahmed, M.A. Sheraz, F.H.M. Vaid, I.A. Ansari, Effect of divalent anions on photodegradation kinetics and pathways of riboflavin in aqueous solution, Int. J. Pharm. 390 (2010) 174–182.
[35] C.K. Remucal, K. McNeill, Photosensitized amino acid degradation in the presence of riboflavin and its derivatives, Environ. Sci. Technol. 45 (2011) 5230–5237.
[36] Y. Yu, L. Yang, C. He, S. Tai, L. Zhu, C. Ma, T. Yang, F. Cheng, X. Sun, R. Cui, S. Wang, D. Wang, An experimental study on riboflavin photosensitization treatment for inactivation of circulating HCT116 tumor cells, J. Photochem. Photobiol. B 196 (2019).
[37] A.V. Juarez, L.D.V. Sosa, A.L. De Paul, A.P. Costa, M. Farina, R.B. Leal, A.I. Torres, P. Pons, Riboflavin acetate induces apoptosis in squamous carcinoma cells after photodynamic therapy, J. Photochem. Photobiol. B 153 (2015) 445–454.
[38] R.A. Akasov, N.V. Sholina, D.A. Khochenkov, A.V. Alova, P.V. Gorelkin, A.S. Erofeev, A.N. Generalova, E.V. Khaydukov, Photodynamic therapy of melanoma by blue-light photoactivation of flavin mononucleotide, Sci. Rep. 9 (2019).
[39] P.F. Heelis, The photophysical and photochemical properties of flavins (Isoalloxazines), Chem. Soc. Rev. 11 (1982) 15–39.
[40] J.N. Chacon, J. McLearie, R.S. Sinclair, Singlet oxygen yields and radical contributions in the dye- sensitized photo-oxidation in methanol of esters of polyunsaturated fatty- acids (oleic, linoleic, linolenic and archidonic), Photochem. Photobiol. 47 (1988) 647–656.
[41] R.W. Redmond, J.N. Gamlin, A compilation of singlet oxygen yields from biologically relevant molecules, Photochem. Photobiol. 70 (1999) 391–475.
[42] A. Żądlo, J.M. Burke, T. Sarna, Effect of untreated and photobleached bovine RPE melanosomes on the photoinduced peroxidation of lipids, Photochem. Photobiol. Sci. 8 (2009) 830–837.
[43] M. Rozanowska, J. Jarvis-Evans, W. Korytowski, M.E. Boulton, J.M. Burke, T. Sarna, Blue light-induced reactivity of retinal age pigment. In vitro generation of oxygen-reactive species, J. Biol. Chem. 270 (1995) 18825–18830.
[44] A. Żądlo, M.B. Rozanowska, J.M. Burke, T.J. Sarna, Photobleaching of retinal pigment epithelium melanosomes reduces their ability to inhibit iron-induced peroxidation of lipids, Pigment Cell Res. 20 (2007) 52–60.
[45] A. Keszler, B. Kalyanaraman, N. Hogg, Comparative investigation of superoxide trapping by cyclic nitrone spin traps: the use of singular value decomposition and multiple linear regression analysis, Free Radic. Biol. Med. 35 (2003) 1149–1157.
[46] Y. Noda, M. Mankura, Inhibitory effect of antioxidants on hydroxyl radical generation from methylguanidine: an ESR study, Neurochem. Res. 34 (2009) 734–738.
[47] B. Kalyanaraman, E.G. Janzen, R.P. Mason, Spin trapping of the azidyl radical in azide/catalase $H_2O_2$and various azide/peroxidase H_2O_2 peroxidizing systems, J. Biol. Chem. 260 (1985) 4003–4006.
[48] L. Skalniak, M. Smejda, A. Cierniak, A. Adamczyk, P. Konieczny, E. Madej, A. Wolnicka-Glubisz, p38 but not p53 is responsible for UVA-induced MCPIP1 expression, Mech. Ageing Dev. 172 (2018) 96–106.
[49] R. Huang, E. Choe, D.B. Min, Kinetics for singlet oxygen formation by riboflavin photosensitization and the reaction between riboflavin and singlet oxygen, J. Food Sci. 69 (2004) C726–C732.
[50] I. Ahmad, S. Ahmed, M.A. Sheraz, F.H.M. Vaid, Effect of borate buffer on the photolysis of riboflavin in aqueous solution, J. Photochem. Photobiol. B 93 (2008)

82–87.
[51] M.A.J. Rodgers, P.T. Snowden, Lifetime of O_2 ^1D_g in liquid water as determined by time-resolved infrared luminescence measurements, J. Am. Chem. Soc. 104 (1982) 5541–5543.
[52] F. Wilkinson, W.P. Helman, A.B. Ross, Rate constants for the decay and reactions of the lowest electronically excited singlet-state of molecular-oxygen in solution - an expanded and revised compilation, J. Phys. Chem. Ref. Data 24 (1995) 663–1021.
[53] C.M. Krishna, S. Uppuluri, P. Riesz, J.S. Zigler, D. Balasubramanian, A study of the photodynamic efficiencies of some eye lens constituents, Photochem. Photobiol. 54 (1991) 51–58.
[54] R.D. Hall, C.F. Chignell, Steady-state near-infrared detection of singlet molecular oxygen:a stern-Volmer quenching experiment with sodium azide, Photochem. Photobiol. 45 (1987) 459–464.
[55] H. Cui, H.M. Hwang, S. Cook, K. Zeng, Effect of photosensitizer riboflavin on the fate of 2,4,6-trinitrotoluene in a freshwater environment, Chemosphere 44 (2001) 621–625.
[56] H. Cui, H.M. Hwang, K. Zeng, H. Glover, H.T. Yu, Y.M. Liu, Riboflavin- photo-sensitized degradation of atrazine in a freshwater environment, Chemosphere 47 (2002) 991–999.
[57] A.B. Ormond, H.S. Freeman, Dye sensitizers for photodynamic therapy, Materials 6 (2013) 817–840.
[58] J. Baier, T. Maisch, M. Maier, E. Engel, M. Landthaler, W. Baumler, Singlet oxygen generation by UVA light exposure of endogenous photosensitizers, Biophys. J. 91 (2006) 1452–1459.
[59] P.C. Joshi, Ultraviolet radiation-induced photodegradation and $^1O_2$, O_2- production by riboflavin, lumichrome and lumiflavin, Indian J. Biochem. Biophys. 26 (1989) 186–189.
[60] E. Sikorska, M. Sikorski, R.P. Steer, F. Wilkinson, D.R. Worrall, Efficiency of singlet oxygen generation by alloxazines and isoalloxazines, J. Chem. Soc. Faraday Trans. 94 (1998) 2347–2.
[61] R. Huang, H.J. Kim, D.B. Min, Photosensitizing effect of riboflavin, lumiflavin, and lumichrome on the generation of volatiles in soy milk, J. Agric. Food Chem. 54 (2006) 2359–2364.
[62] M. Schuman Jorns, G. Schollnhammer, P. Hemmerich, Intramolecular addition of riboflavin side-chain - anion-catalyzed neutral photochemistry, Eur. J. Biochem. 57 (1975) 35–48.
[63] W.M. Moore, J. Baylor, The photochemistry of riboflavin. IV. The photobleaching of some nitrogen-9 substituted isoalloxazines and flavins, J. Am. Chem. Soc. 91 (1969) 7170–7179.
[64] M. Munoz, A. Pacheco, M.I. Becker, E. Silva, R. Ebensperger, A.M. Garcia, A.E. De Ioannes, A.M. Edwards, Different cell death mechanisms are induced by a hydro-phobic flavin in human tumor cells after visible light irradiation, J. Photochem. Photobiol. B 103 (2011) 57–67.
[65] C.A. Rappole, K. Mitra, H. Wen, Dynamic fluorescence imaging of the free radical products of X-ray absorption in live cells, Opt. Nanoscopy 1 (5) (2012) 1–9.
[66] M. Wrona, K. Patel, P. Wardman, Reactivity of 2′,7′-dichlorodihydrofluorescein and dihydrorhodamine 123 and their oxidized forms toward carbonate, nitrogen di-oxide, and hydroxyl radicals, Free Radic. Biol. Med. 38 (2) (2005) 262–270.