SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of cell biology effect of a promising photodynamic small molecule, diMICAAc

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DEBRECEN, 2022

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The Examination takes place at Life Science building 5.306 Faculty of medicine, University of Debrecen, 2019.12.16.

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2022.09.02. 13:00

1. Introduction

1.1 The rise of photodynamic therapy

According to WHO statistics, malignant lesions of various origins, such as trachea, bronchus, lung stock, colon, rectum and even brain tumors are among the leading causes of death in europe and globally among all men and women. The immune system's defenses against pathogens can also recognize and remove the body's own aging, damaged or dead cells, while the immune system does not act against healthy tissue cells, but remains "tolerant". The possibility of developing tumors proves that the immune system does not or always perform this task properly. After the diagnosis of tumors, the most significant therapeutic options include surgical, radial (or radio) and chemotherapy treatments. Depending on the type of tumor, these therapeutic options are used either as a standalone therapy or in combination to eliminate the tumor. Most established chemotherapy drugs act in a DNA-damaging way, leading to the apoptotic destruction of tumor cells. Canonical apoptosis is part of normal tissue regeneration, and thus physiological homeostasis. Immunologically quiet process, does not induce pathological danger signs. The activation of apoptosis tends to produce an anti-inflammatory effect, so the apoptonic destruction of cancer cells often goes unnoticed by the immune system. At the same time, in the case of pathological cell destruction processes that occur when tumor cells are formed, specific immune cells can recognize the danger and activate the immune system. The effective immune response to cancer cells bears many similarities to cellular immune secrets induced by cells infected with the virus, insofar as the most effective executing/effector cells of both the viral and antitumor immune responses are natural killer cells (NK cells) and cytotoxic T lymphocytes (Tc). The two types of cells distinguish differently between healthy and altered cells, virus-infected or cancerous cells with altered properties, but the initiation of cell-destroying mechanisms through the transmission of similar molecules leads to damage to the membrane of the membranes of cells that carry the virus or cancer and destroy the target cell. These strictly regulated processes are initiated against pathological conditions, which ultimately leads to the destruction of cells. This method of cell death is called immunogenous cell death. The condition of immunogenic cell death is the permeability of the plasma membrane and the release of some intracellular damage-related molecules (DAM). ATP, the protein of the endoplasmic reticulum, is calreticulin, HMGB1, the DNA-binding nuclear protein, and the RNA and DNA of cells are typical DAMs.

Nowadays, an increasing number of people are using the so-called photodynamic therapy (PDT), which is located on the border between biological therapy, chemotherapy and radiotherapy. With PDT, it can make a diagnosis in one step and therapeutic procedure, called teranostics. The essence of PDT is to inject an atoxic compound into the body that alone does not have any cell damage effect. It is well known that visible light belongs to electromagnetic waves, the wavelength range of which is between 400 and 750 nm, and visible light alone does not cause cell damage to the body. The essence of the procedure, however, is that the injected compound, illuminated in a given area with light of a given wavelength, in the illuminated areas, the cells die due to various direct and indirect effects.

1.2 PDT mechanism and molecular background

PDT is a noninvasive therapeutic procedure that can affect tumors, bacteria, viruses and microbes. During therapy, three things play a key role in triggering the effect: a photosensitizing substance, light, and O_2. The cells absorb the photosensitizing substance, which, in the absence of light, is harmless to both healthy and malignant cells. However, when illuminated with light of a given wavelength and the photosensitizing material absorbs a photon, it moves from a stable base state to a short-lived excited state. From there, in a short period of time, an internal conversion (IC) is transferred to the lower vibrational level of the first excited state through heat dissipation. There are several options for returning to the basic state, which can occur with varying probabilities depending on the environment or molecule. When the photosensitive was illuminated with the right wavelength light and the excited state occurred, two biological outcomes are possible from the T1 state. In a type I reaction, the excited molecule reacts with a substrate and reactive radicals are formed e.g. (H_2O_2, O_2, OH) that can react with proteins (tryptophan, tyrosine, histidine, cysteine) lipids (unsaturated phospholipids and colesterol) and nucleobases (guanin). It does not interact with carbohydrates.

In the case of a type II reaction, the photosensitizing material transmits energy to oxygen and oxygen is produced from a triplet state of oxygen in a singlet state. After that, reactive radicals or the singular state of O_2 induce an oxidative stress response in the cell, to which the cell gives a cellular response, and if the change is such that the cell can no longer compensate for the effect (irreversible) the cell dies. There are several types of cell death that can occur during PDT therapy, depending on what kind of photosensitive, what target organism is used against. In addition to the commonly known programmed cell death (apoptosis) and sudden cell necrosis, autophagy and paraptosis also play a role.

1.3 Generations of photosensitives and PDT

Among the most common photosensitives are porfin vase compounds. However, there are several types of photosensitives used in PDT. These subtypes are divided into different generations. First-generation photosensitives were successfully used for the first time in the early 20th century. In the case of second-generation photosensitives, the development was aimed at increasing strength and efficiency. Another goal was to be successfully used in as many diseases as possible. The research focused on increasing absorption wavelengths, because by increasing the wavelength, tissues have less extinkation coefficient in the red/near infrared range of the electromagnetic spectrum (600-850nm), which allows the light used for illumination to penetrate deeper into the skin. And by increasing the depth, it is possible to increase the availability of tumors. In the second generation, the spectrum of photosensitives widened. Porphyrins remained in clinical use, but chlorine, texapirin, porphycin, purpurins and phtalocyanins were introduced. In third-generation photosensitives, research focuses on the possibility of injecting the drugs directly into the bloodstream, unlike first and second generation photosensitives with low solubility. The compounds are developed for direct targets and photosensitive substances can be used either by attaching antibodies or injected into nano carriers as a targeted biological therapeutic agent. It is also important to increase the effectiveness of therapy by increasing selectivity and specificity. Today, the most important principle during PDT is to be able to diagnose in one step and also apply a therapeutic procedure in the shortest possible time with the greatest efficiency. With the development of imaging, it is possible to use the fluorescent properties of photosensitives to more accurate localization, and with pharmacokinetics and pharmacodynamic knowledge, it is possible to use small molecule therapeutics.

1.4 Wide-ranging biological function and photodynamic therapeutic effect of acryridin vase compounds

Acridine vase compounds have a large number of biological activity, excellent pH indicators, also have antimalaria and anti-tumor effects. It is also used to identify bacteria and parasites, in addition, they are also suitable for the examination of apoptosis and sperm motility. One of the most characteristic compounds is acridine orange (AO), which was first extracted from coal tar as a weak base dye more than 100 years ago. AO has also been used as a photosensitive for several years, as its effect is the same as the most common photosensitives, porfin vase mixtures. Therefore, it plays an important role in PDT therapy. AO has a low molecular weight, can quickly diffuse from the cytoplasm to the nucleus of the cell in just a few seconds, where it binds to DNA and RNA, but it can also get into lysosomes and other acidic vesicles at the same speed. AO is able to emit a green fluorescent signal after blue light excitation, allowing macroscopic examination of tumors. AO has not only been successfully tested under in vitro conditions, but has also had successful clinical uses. After the synthesis of acridine vase compounds, different functional group exchanges can create compounds with different properties that can enhance the photodynamic effect. One possible solution for the production of "smarter" fluorescent paint could be to replace one of dimethyl amino groups with a multifunctional electron extraction group, such as isocyanide. Despite the versatile chemistry of isonitriles, the area of isocyanoacridine has so far remained completely undiscovered. The installation of the isocyano group has many advantages: you can reduce the value of an pKa from 10.5 to around 7.0, so a powerful, reactive C-N bond can serve as a versatile base for many organic reactions. Isonitrils easily form complexes with transient metal ions. 3-amino-6-isocyanoacridine (ICAAc) can be obtained by treating proflavin with dichlorocarben, as a result of which one amino group is converted into izonitrille. At the Department of Applied Chemistry, three new compounds were synthesized:

1. *3-amino-6-isocyanoacridine (**ICAAc**),*
2. *3-N-metilamino-6- isocyanoacridine (**monoMICAAc**)*
3. *3-N,N-dimetilamin-6-isocyanoacridine (**diMICAAc**)*

In diMICAAc (DM) there is a polar isocyanate function group that replaces the apolar dimethylamino group in AO. The negatively charged end of the polar segment of the DM is outward-oriented. Compounds with a functional group of isocyanate have been proven to be used as pH probes of lysosomes in physiological cellular conditions.

2. Goals

PDT agents are increasingly being used in a wide range of applications, both diagnostic and therapeutic. Such potential PDT agents are acridine scaffolds synthesized by the Department of Applied Chemistry, University of Debrecen. We aim to better understand the physical and chemical properties of these new compounds, to find out which of the three newly synthesized compounds are suitable for the treatment of living cells under non-illuminated conditions, and to compare the phototoxicity properties of the previously selected agent(s) with the cellular phototoxicity of acridine orange, a compound that is well characterized in the literature.

We have set the following objectives:

- We want to know the relevant optical properties of the newly synthesized compounds, e.g. absorption and emission spectra. We want to perform these experiments using spectrofluorimetry.
- Several acridine skeletal compounds are known to be toxic, which rules out their use in PDT. Therefore, it is necessary to determine the toxicity of these compounds. We want to determine the toxicity by MTT method.
- We want to understand the intracellular distribution of fluorophores and their kinetics. We plan to use confocal and laser scanning cytometry for kinetic experiments.
- We want to determine the phototoxicity properties of compounds. We want to investigate phototoxicity under both continuous and fractional illumination.
- We also want to study the cellular mechanism of action of phototoxicity.
- Acridine orange has been shown to enter both the lysosomes and the nucleus of cells. We are also curious to know which organelles the new compounds can enter. We also want to determine these experiments using microscopy.
- We want to investigate the effect of compounds on chromatin. We would like to use the QUINESIN method for chromatin level studies.

3. Materials and methods

3.1 Cell lines

Some of the cells used in the experiments (SKBR-3, HeLa, and OCM-1) were obtained from the American Type Culture Collections (ATCC, Manassas, VA), while 3T3-MDR (NIH 3T3 MDR1 G418) was given as a gift from M. Gottesman. SKBR-3 is a trastuzumab-sensitive human breast tumor, HeLa is a human cervical cancer, OCM-1 is of human melanoma cell line (ocular choroidal melanoma), while 3T3-MDR, an MDR1 gene is a transducible mouse fibroblast cell line. The cells were growing according to their specifications in DMEM or RPMI containing 10 % fetal calf serum (FCS, BioTech, Budapest, Hungary), 2 mM of glutamine (Sigma Aldrich, Budapest, Hungary), 10 U/ml penicillin (Sigma Aldrich, Budapest, Hungary) and 10 μg/ml Streptomycin (Sigma Aldrich, Budapest, Hungary) or gentamycin (50 μg/ml) and phenol red indicator (LiveTech, Montizello, Italy) up to an early flunx state at 37 ^{o}C in the presence of 5 % CO_2. Each cell line was grazed every two days, with HeLa and OCM-1 cells in the medium RPMI medium, while SKBR-3 and 3T3-MDR were maintained in the medium DMEM. The cells were tested for Mycoplasma.

3.2 Comparison of fluorescent properties of ICAAc, monoMICAAc and diMICAAc with laser scanning cytometry (LSC)

In the first phase of the experiments, HeLa cells were plated in an 8-well IBIDI (Ibidi, Martinsried, Germany) chamber. The cell number was 30.000 cells/cm^2. We first wanted to see if the three compounds were taken up by the cells. ICAAc, monoMICAAc and diMICAAc were added to the cells at final concentrations of 1.25×10^{-6} M and 2.5×10^{-6} M, respectively. Propidium iodide was added to the cells at a final concentration of 7.5×10^{-6} M. Using a laser scanning cytometer, 4x4 field images were obtained for both 1.25×10^{-6} M and 2.5×10^{-6} M concentrations after 30 min incubation. The resolution was 1024x768 pixels. The width of the square pixels was 0.25 μm. After 488 nm laser excitation, a 40x (0.75 NA) objective was used for the measurement. The signals were detected in three channels (blue, green and far red). Subsequently, images of the cells were repeatedly acquired at a concentration of 1.25×10^{-6} M in the acute and after one hour of incubation. For quantitative determination, compounds were added to the cells at a concentration of 1.25×10^{-6} M. Also in 8-well IBIDI, HeLa cells were cultured at 30.000 cells/cm^2. After thirty minutes of incubation, a 4x3 field mosaic image of the cells was obtained. The resolution of an image field was 1000x768 pixels, X-pixel width: 0.25

μm. After excitation at 488 nm, a 40x (0.75 NA) objective was used for the measurement. The signals were detected with an emission filter at 530±15 nm. To examine the fine structures of the cells, HeLa cells were also cultured in 8-well IBIDI at a cell number of 30.000 cells/cm^2. After incubation at 1.25×10^{-6} M for 30 min, the cells were imaged. An Olympus IX-71 inverted fluorescence microscope camera was used to take the images. Images were acquired in transmission, blue, green and red emission channels.

3.3 Determination of toxicity

Toxicity determination was carried out on HeLa cells. In the case of AO, the starting molar concentration was 3.62×10^{-5} M, in the case of DM it was 3.88×10^{-5} M, which were then diluted in 10 different halve concentrations. In both cases, the treatment time was 24 hours. After the incubation period, MTT (Sigma Aldrich, dissolved in 0.5 mg/ml PBS) was added to the cells. 100 μl of MTT solution was inserted into each hole. At that time, the incubation period was 2 hours at 37 °C in the presence of 5 % CO_2 in a cell breeding incubator. The formazan crystals were dissolved with 200 μl DMSO per hole. The incubation period of DMSO was half an hour, also at 37 °C in the presence of 5 % CO_2 in a cell breeding incubator. When the crystals dissolved synergy HT automatic ELISA Plate Reader, we measured absorbance at 490 nm and 620 nm. The absorption values were determined by the formulas × (absorption of treated cells - control cells). Values above 0.5 were characteristic of living cells, while values below 0.5 were characteristic of dead cells.

3.4 Spectrofluorometric measurements

UvVis spectra was recorded with a Jasco FP-8200 spectrophotometer. The device has an Xe lamp. For the measurements, quartz quartz cuvettes with an optical length of 1 cm were used. The excitation and emission spectra were recorded at a temperature of 20 °C at a width of 2.5 nm and an emission band of 5.0 nm. The panning speed was 200 nm/min with normal sensitivity" Pipettes of 3 ml of solution were pipetteized into each cuvette. 2.26×10^{-6} M molar concentration AO and 2.43×10^{-6} M DM molar concentration solutions were used. During the experiment, solutions were added to HeLa cells at the concentration described above. The number of HeLa cells was 1 million/ml. In a cellular case, we determined both the excitation and emission spectrums. Using the same settings, we recorded the spectra in a case without cells and looked at whether there was a difference between the two measurement results.

3.5 Determination of emission spectra by Zeiss LSM 880 confocal microscope

Twenty-four hours before the experiment, we plated the cells to an 8-hole IBIDI plate. The cell count was 30.000 cells per hole. We treated the cells with 2.26 $\times 10^{-6}$ M AO molar concentration solution and DM solutions with a molar concentration of 2.43 × 10-6 M. The treatment time was 2 hours. After treatment, we used a Zeiss LSM 880 confocal microscope to take 16-bit images of the cells, we recorded the images in 4 channels, with light microscopic illumination, excitation of 405 nm, 488 nm and 543 nm. The master voltage in the first channel (488 nm): 830, in the second channel (transmission image): 672, in the third channel (405nm): 850 and in the fourth channel (543 nm): 850. Digital amplification was 1.0, while digital offset was 0.00 in all channels. In the 488 nm channel, the pinhole was 42 μm, in the 405 nm channel it was 31 μm, and in the 543nm channel it was also 42 μm. The following filters were used: 499-695 nm, 410-474 nm, 554-629 nm. Laser intensities were 0.02 % for 488 nm, 1.8 % for 405 nm and 1.5 % for 543 nm. The same settings were used when determining the fine structures of the cells and when examining the spectra, but in terms of better visibility of the images, we used a median filter 2.0 filter and increased contrast and brightness by 60%.

3.6 Determination of lysosome activity with Zeiss LSM 880 "Lysotracker Orange"

We bred HeLa cells on 3 8-hole IBIDI plates 12 hours before the measurement. The cell count was 30.000 cells per hole in each hole. We treated the cells with 2.26 $\times 10^{-6}$ AO molar concentration solutions and DM solutions with a molar concentration of 2.43 × 10^{-6} M. The incubation period was 1 hour in a cell breeding incubator at 5 % CO_2 at 37 °C. For positive control, the cells were treated for 30 minutes with "Lysotracker Orange" (ab176827, CytoPainter, LysoOrange Indicator Reagent, abcam) under similar conditions. We put 20 μl "Lysotracker Orange" in 10 ml of RPMI medium and put 300 μl of them in the holes and after 30 minutes we took pictures of the cells. The excitatory laser was 543 nm. In the case of cells treated with AO and DM, after 1 hour of incubation, we selected a cell and took a picture of it. This was the picture before the lighting. We then took 1,000 images (50s) of the same cell and made a video of the images, which were taken at 20 frames/sec. Then we again took an image of the cell, which reflected the state after illumination. After the image after the illumination, we carefully drained the liquid and added "Lysotracker Orange" diluted in a medium of 300 μl. It was a very important step that we had to carry out both the suction and the addition of fluid with due care, because it was not allowed for the cell to move. After that, 15 minutes of

incubation followed at 37 °C. After 15 minutes of incubation, we took a picture of the illuminated cell and the cells around it. This condition mirrored the lysosome activity after illumination in both the illuminated cell and its surroundings. This process was repeated after both AN and DM treatments. Excitation was 405nm, 488nm and 543 nm. Digital "gain" was 1.0 and digital "offset" was 0.00 in all channels. The emission filters were: 499-695 nm, 410-474 nm, 554-629 nm. The laser intensity was 0.02 % for 488 nm, 1.8 % for 405 nm and 1.5 % for 543 nm excitation.

3.7 LED induced phototoxicity test

To determine the kinetics of phototoxicity, LED induced lighting was used. In the experiments, we put HeLa cells in 96-hole breeding plates with 10.000 cells per hole. We treated the cells with 2.26×10^{-6} AO molar concentration solutions and DM solutions with a molar concentration of 2.43×10^{-6} M. The lighting times were 0 hours, 1 hour, 3 hours, 8 hours and 24 hours, during which treatment times we illuminated the cells with 470 nm blue and 525 nm LED lamps. At the time of illumination, the lamp and breeding vessels were placed in a cell breeding incubator at 37 °C with a CO_2 level of 5 %. The height of the lamp was 10 cm measured from the 96-hole plate. The light diameter was 6 cm. As a control of the treatments, a 1 % DMSO solution was used. After the illumination times, we determined the cell viability by measuring MTT. 100 µl MTT (Sigma Aldrich 0.5 mg/ml concentration dissolved in PBS) solution was inserted into each hole. At that time, the incubation period was 2 hours at 37 °C in the presence of 5 % CO_2 in a cell breeding incubator. The form crystals were dissolved with 200 µl DMSO per hole. The incubation period of dissolution in DMSO was half an hour also at 37 °C in the presence of 5 % CO_2 in the cell breeding incubator. When the crystals dissolved synergy HT automatic ELISA Plate Reader, we measured absorbance at 490 nm and 620 nm. The absorption values (absorption of treated cells -- absorption of control cells) were determined by × 100 formulas. Values above 0.5 were characteristic of living cells, while values below 0.5 were characteristic of dead cells.

3.8 Determination of cell viability with laser scanning cytomometer

HeLA cells were placed 24 hours before the experiments in 8-hole IBIDI breeding plates in 30.000 cell counts. We treated the cells with 2.26×10^{-6} AO molar concentration solutions and DM solutions with a molar concentration of 2.43×10^{-6} M and we added propidium iodide to

each sample at a final concentration of 7.5 × 10-6 M. We designated a 2x2 ROI and took pictures of the same areas in 63 cycles. The length of 1 cycle is 5 minutes, so the total measurement time was 5 hours 15 minutes. The size was 1024x768 pixel. The X-step size was 0.5 μm. The reflected fluorescent signals were detected in 3 channels (green, red and distant red) with a 40x LWD (NA 0.6) lens. We used 405 nm, 488 nm and 633 nm lasers to excite. The detected signals have been converted into 14-bit high-resolution images. After the LSC measurement, mercury vapor lamp recordings of the given samples were taken using CellB. We used a 40x lens (0.75 NA). The exposure time was 100 ms for a light microscope and 588 ms for the other filters with ISO 200.

3.9 Histonian method of elution

Before embedding, the holes in the 8-hole chambers (Ibidi, Martinsried, Germany) were coated with a 1 % low melting point agarose. For the time of embedding, both the cells and the agarose were kept at 37 °C. 6×10^6 cells were mixed in 1x PBS (150 mM NaCl, 3,3 mM KCl, 8.6 mM Na 2 HPO 4, 1,69 mM KH_2PO_4, pH 7,4) diluted 1 % LMP agarose in a ratio of 1:3. (Fellowes, Inc., Itasca, Illinois, USA). The cells were kept at 37 °C for 4 minutes so that they could settle on the surface of the coated holes. After polymerizing the agarose, we put 300 μl of ice-cold medium in each hole to help remove the plastic lid. After the embed, a 2x2-sized ROI was illuminated in each hole with a laser scanning cytomet. We treated the cells with 2.26×10^{-6} AO molar concentration solutions and DM solutions with a molar concentration of 2.43×10^{-6} M. The lighting time was 2 hours. We used a 488nm laser, a 40x LWD lens and an X-step size of 0.5 μm for illumination. Cells embedded in LMP agarose were washed in an ice-cold 1x PBS (500 μl for each hole) for 3x3 minutes. We then permeabilized the cells with a 500 μl ice-cold permeabilizing solution in which the deterrent Triton X-100, 1% (v/v), dissolved in 1xPBS/EDTA (5 mM EDTA PBS). The treatment time was 10 minutes on ice. This step was repeated once more in each hole. After permeabilation, the nuclei were washed for 3x3 minutes with 500 μl ice-cold 1x PBS/EDTA. (500 μl for each hole) After that, for 60 minutes, we treated the seeds with NaCl with different concentrations (0 mM, 100 mM, 600 mM, 700 mM, 800 mM, 900 mM, 1000 mM and 2000 mM). The next step was a 3x3 minute wash with 1x PBS/EDTA. (500 μl for each hole). After NaCl treatment, a blocking buffer was added to the cells, 500 μl in each hole, in which 5 % (m/v) Blotto Non-Fat Dry Milk (Santa Cruz Biotechnology Inc., Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) milk powder was dissolved in 1x PBS/EDTA. To prevent nonspecific attachment, the blocking step was 30

minutes. After blocking, the nuclei were washed with 500 µl of ice-cold 1x PBS/EDTA for 3x3 minutes (500 µl for each hole). This was followed by indirect antibody marking, in which a polyclonal rabbit IgG anti-H2A (Abcam, Cambridge, UK; 0.4 mg/ml, ab 18255) antibody was used as a primary antibody. The antibody was diluted in 150 µl 1xPBS/EDTA/1 % BSA (1xPBS/EDTA which contained 1 % calf serum albumin, 1:800 titres). The marking was made at 4 °C throughout the night. After primary antibody marking, a 3x10-minute wash was followed by 1x PBS/EDTA. (500 µl for each hole). As a secondary antibody we used Alexa Fluor 647 conjugated goats produced anti-rabbit IgG (Thermo Fisher Scientific, Waltham, Massachusetts, USA, 1:800 titres, dissolved in 1xPBS/EDTA from a strain concentration of 2mgP/ml). The marking was made on ice for 2 hours. After secondary antibody marking, a 3x10 minute wash followed with 1x PBS/EDTA. (500 µl for each hole). After marking, the samples were fixed with a fixative solution with a final concentration of 1 % formaldedide (dissolved in PBS) at 4 °C all night. After fixation, a 3x3 minute wash followed with 1x PBS/EDTA. (500 µl for each hole). After washing, we painted the seeds with propidium iodide with a final concentration of 5 µg/ml (PI, dissolved in 1×PBS/EDTA) on ice for 30 minutes. After pi painting, a 3x3 minute wash followed with 1x PBS/EDTA. (500 µl for each hole). Fluorescent intensities were measured on an iCys laser scanning cytometer. With a 20x lens (0.17 NA), a size of 0.5 µm X-step, a ROI size of 10x7, all samples were measured. We evaluated the samples with ICys 7.0 software.

3.10 Quantitative determination of lysosomes with ELISA HQ Plate Reader and LSC

Quantitative determination of lysosomes was carried out on HeLa cells. In the case of AO, the starting molar concentration was 3.62×10^{-5} M, for DM it was 3.88×10^{-5} M, which were then diluted in 10 different halve concentrations with 4 pair-row measurements, positive control was given RPMI medium and 0.7 % glucose PBS was added to the cells in 4-4 cases. In the negative case, there was also RPMI and glucose PBS in the holes, but there were no cells. Another treatment that could be classified as negative control was LLoMe (L-leucyl-L-leucine methyl ester, Sigma Aldrich, 25nM cc.), which was added to 8 holes. The LLoMe literature has been shown to reduce lysosome activity. After the addition of compounds, the incubation period was 45 minutes in a cell breeding incubator at 37 °C at a CO_2 level of 5 %. After 45 minutes of incubation, we measured the fluorescent intensities of the samples using the BioTek Synergy HT fluorescent microplate reader. The excitation filter was 485/20 for both AN and DM. The emission filter was 528/20 nm for AN and 590/20 nm for DM. After that, we measured the

intrusions 10 times in each hole, illuminated from above. After illumination, we drained the liquid from the cells and added "Lysotracker Orange" diluted in a medium of 300 µl. Dilution was carried out in the manner described above. After 30 minutes of incubation, we took a photo of each hole with an Olympus DP71 camera. To extract the ratio of lysosomes, we used classic image processing methods.

As a first step, we performed image correction procedures on the input images. In addition to filtering visual noise, we performed image sharpening using a procedure called "unsharp masking" to achieve more efficient segmentation. After the image correction, the cells were segmented. During segmentation, a binary image is formed, on which the object and the background are separated, that is, during the procedure, we carried out the separation of cells from the background. Eliminating overlaps and small impurities in the sample required a complex segmentation process. In the first step, the separation of cells and background was carried out using adaptive threshold algorithm. The result is a slightly noisy and sometimes incomplete segmented binary image with holey regions. To correct these errors, we used the so-called morphology operations. Morphological operations are suitable for correcting segmentation errors and are the preparation of the image for shape recognition. To remove small noises, we used the "erode" morphology operator, with which it is possible to eliminate the points assigned to the object in the background area, and smaller shapes; It is also suitable for separating merged objects. In addition to the erode operator, the opening morphology operation was also used for a similar purpose, keeping the size of objects (cells) in mind. Even after segmentation, as well as erode and opening operations, holes can form at the edges and inside objects. We used the morphological operator "dilate" to patch these holes. The size of the structuring element used by the operators is determined individually for each image, depending on the degree of noise and stains generated.

4. Results

4.1 Comparison of fluorescent properties of ICAAc, monoMICAAc and diMICAAc with laser scanning cytometry (LSC)

Comparing acute and post 30 min incubation images, it can be concluded that the intensity of microscopic fluorescence within diMICAAc cells is around 50, while ICAAc is 3 and monoMICAAc has an average fluorescence intensity of around 5. That is, diMICAAc has at least one order of magnitude higher microscopic fluorescence intensity compared to the other two paints. This can be caused by the fact that the other two substances are not absorbed by the cells in the same amount as diMICAAc, or as a paint molecule diMICAAc is a more stable compound than the previous two. It may also explain that diMICAAc diffuses more quickly into the cytoplasm and then into the target organellam faster than the other two compounds. Transmission images clearly show that the cell morphology is the most beautiful for diMICAAc, so diMICAAc is probably the least toxic of the three substances. ICAAc and monoMICAAc are not effective enough in marking living cells, no microscopic emissions are visible at excitations with wavelengths higher than UV, whereas diMICAAc is. ICAAc is more attached to the cell membrane. MonoMICAAc may be quite faint, but it can label DNA by entering the nucleus of the cell and, in the cytoplasm, probably by binding to proteins, to denote cytoplasm. DiMICAAc, on the other hand, intensively paints subcellular organelles. The most intensely visible nuclei become visible, so this compound is able to enter the nucleus and bind to DNA or RNA there. At the same time, the pattern of painting accumulated in the lysosomes visible in diMICAAc.

4.2 Toxicity

The results obtained show that acryridin oranges are more toxic in certain concentrations than diMICAAc, but there is no significant difference between the two compounds. The LD50 toxicity of the paints was 4.4×10^{-6} M for AO, while 4.01×10^{-6} M for DM. The 95% confidence levels are AO: 3.43×10^{-6} - 5.76×10^{-6} M, DM: 3.33×10^{-6} - 4.83×10^{-6} M. Since AO has been removed from the tumor-causing compounds by the WHO, it can therefore be used as a photodynamic therapeutic agent. Furthermore, in terms of their cellular toxicity, the two compounds behave very similarly, so diMICAAc (DM) may also be suitable for therapeutic procedure based on its cellular toxicity.

4.3 Spectrofluorometric measurements

In our results, we do not see wavelength shifts compared to cellular and cellless spectra. The local absorption maximum in both cases was 488 nm. In the presence of cells, there was a significant increase in light scattering. By examining the emission spectra, we can conclude that in the case of AO the emission maximum is at 530 nm, whereas in the case of DM the maximum is shifted to the right, at about 570 nm. There is no shift in comparison with cases without cells and cells. Reading the scales also shows that AO has a higher fluorescence efficiency than DM.

4.4 Determination of emission spectra by Zeiss LSM 880 confocal microscope

From the emission spectrum obtained after 405 nm excitation of spectral imaging, it can be stated that the emission spectrum of DM is wider than that of AO. The emission maximum is shifted to the right in case of DM. It can also be observed that different cells have taken up the compounds to varying degrees. SKBR-3, OCM-1 and HeLa cells were taken up to a greater extent, while in the case of 3T3-MDR, it is likely that the paints were absorbed in smaller quantities thanks to the transporter P-glycoprotein ABC, as this transporter with a broad hydrophobic molecular spectrum pumped them out into the extracellular space. AO and DM excite better at 488 nm than after 405 nm excitation. The emission maximum is also shifted to the right for the DM. Here, too, it can be observed that the cells took up the compounds in different quantities. AO usually label cells better at this wavelength. The compounds were taken up more in order by the SKBR-3, OCM-1 and HeLa cells, while in the case of 3T3-MDR, less paint was able to get in due to P-glycoprotein. DM is better excited from 543 nm than AO.

4.5 Determination of the fine structures of cells using a Zeiss LSM 880 confocal microscope

Using settings similar to spectral imaging, we also examined the delicate structure of cells. DM can be excited at 405 nm and here we can identify small vesicles in the image, which are different from the nuclei visible by excitation of 488 nm. We think that these are acidic vesicles and lysosomes. If we compare the two substances after excitation of 488 nm, we can conclude that AO clearly entered the nucleus. In the case of DM, the cytoplasm is painted evenly, and in the nucleus the nucleus the nucleus appear intensely, but the contour of the nucleus can also be faintly visible. It is known from the literature that with excitation of 543 nm, lysosomes and other acidic vesicules can be displayed at AO in the red emission range, but even cytoplasm can be suspected here, while in the case of DM the pattern is similar to 488 nm excitation.

4.6 Determination of lysosome activity with Zeiss LSM 880 "Lysotracker Orange"

From the images, it can be established that the fluorescent intensity of the illuminated cells is clearly decreasing. After illumination, only the intensity of the illuminated cells decreases, not that of the surrounding cells. Control images clearly show that in unlit cells, “Lysotracker Orange” neatly denotes small vesicule like lysosomas not damaged in its membrane.

4.7 LED induced phototoxicity test

In the case of blue LED lighting, in the case of unlit control, the compounds did not show toxicity in the concentration used for phototoxicity (0 hours of illumination). The figure shows that after 1 hour of illumination, the phototoxic effect of DM occurs sooner. In the case of 3 hours of illumination, a similar phototoxic effect can be seen in both cases, while after 8 hours of illumination, the DM again appears more phototoxic than AO. After 24 hours of lighting, virtually none of them can be observed living cells. In conclusion, increasing the illumination time reduces viability. In the case of green LED slitting, we can see a slightly higher degree of phototoxicity of the DM after 1 and 3 hours of illumination. After 8 hours of illumination, almost all cells die, but here too, after 24 hours of DM. After 24 hours of lighting, the cells are practically completely destroyed.

4.8 Determination of cell viability with laser scanning cytometre

The difference between laser scanning cytometry measurements and LED lantern experiments was the continuity of illumination. While in the case of the use of LED, the illumination was continuous during the treatment time, in the case of LSC there was only illumination at the time of the taking of the images, which in our case meant a illumination time of 2.9 s per cycle. So, in contrast to the continuous illumination of the LED lamp, LSC illumination was fractionally dosed every 5 minutes at the illumination times of 2.9 s per cycle.

The performance of the LSC's 488 nm laser for continuous illumination was 0.30 mW ± 0.05 (average ± SD), which corresponded to a light power of 0.32 W/cm^2 for the sample. In contrast, LED lighting had less light power: 4.45 mW/cm^2.

PI is known to be able to pass through only the membranes of inanimate cells or highly damaged cells. At 0 o'clock, we can see that the fluorescent intensity of AO is higher than that of DM. After 1 hour, in the case of AO, in the middle of the image ("well image" there are a few places where the intensive PI-painting decaying cells begin to appear. At 2 hours in the middle (at the fitting of the "field image" images, where the laser beam passes several times in a cycle), more and more PI positive cells can be observed. At 3 o'clock it is noticeable that in the case of AO, practically all cells die, thanks to the fact that in case of AO fractional illumination, an atoxic compound than DM. from 4 o'clock all cells die in both cases.

4.9 Histonian method of eludence

When the unlit HeLa cells were treated with AO or DM intercalators, the histone H2A proteins were excreted from the nucleus according to a sigma curve pattern as salt concentrations increased. For DM, a higher amount of initial histone protein intensity was measured for concentrations of 100 and 600 mM. In both cases, it can be seen that by increasing the concentration of NaCl, we detected a tendency to decrease amounts of histone protein. This is striking in the case of DM, as an order of magnitude lower amounts of histone remained in the cell after the highest salt concentration treatment in the unlit area. The amount of histone in the DM illuminated area remained the same in the order of magnitude for both the smallest and highest salt concentrations. In the case of AO, the amount of histone protein decreased in both illuminated and unlit areas compared to DM dye, which may mean, on the one hand, in the unlit case, that AO, already attached to the DNA by binding to the DNA, causes the elusion of histonoid proteins, and on the other hand, in the case of illumination, it damages the DNA to a

greater extent than DM. Overall, it can be concluded that after illumination, the histone proteins were attached to chromatin in the case of DM, while in the unlit part, by increasing the salt concentration, the histones could be hated for both DM and AO, since we detected decreasing amounts of histone. In addition, we can conclude that AN causes more intense DNA damage in fractional irradiation than DM.

4.10 Quantitative determination of lysosomes with ELISA HQ Plate Reader and LSC

It can be concluded that by increasing the final concentrations of photosensitives, the number of lysosomes in cells decreased. Compared to the control, the amount of lysosomes decreased for both AO and DM. In case of positive control, lysosome damaging LLOMe positive control is also observed to show reduced lysosome activity. Without cells, only in the case of a medium, no lysosome signal could be detected at all. At a similar molar concentration, DM caused a higher degree of lysosome damage than, an.

5. Discussion

New multifunctional amino-isocyanoacridines (ICAAc, monoMICAAc and diMICAAc) have been produced with a reaction of 3.6-diaminoacridin and dichlorocarbene. The resulting fluorophores exhibited a tuneable solvatoochrome behavior due to the dipole nature of the molecules ($\Delta\mu$ = 5.6-8.9 Debye) ($\Delta\lambda$em = 53-63 nm). The use of the electron-extracting izonitrile group resulted in reduced baseness (pKa=7.05-7.58), which, together with the unique pH-sensitive vibration absorption bands, makes the compounds promising pH probes in a physiological pH range. In addition, the isocyano group is an excellent ligand for complex training with Ag(I), so the optical behavior of our paints can also be improved, even fine-tuned with ions. The applicability of ICAAc, monoMICAAc and diMICAAc (DM) paints for the imaging of living cells was demonstrated on HeLa cells at 2.5×10^{-6} M and 1.25×10^{-6} M in final concentrations. Cells treated with DM showed the best preserved morphology. In the first phase of the experiments, from the images we have created, it can be concluded that DM binds to the cell membrane, can enter various intracellular vesicular structures, is slightly attached to DNA and RNA. All three compounds are well suited for traditional epifluorescent imaging even with UV excitation. Furthermore, in addition to viola blue excitation, DM is excellent for cell pH probes for epifluorescens and laser illumination, such as flow and imaging cytometry or confocal laser scanning studies.

We could see from the experiments that DM seemed to be the most promising compound of the 3 compounds, so in the further phase of the experiments we compared this fluorophore with a substance already extremely accurately characterized in the literature, AO. To clarify how this simple chemical modification, which we have already demonstrated, changed the properties of DM fluorescence, which can be used in cellular applications, we compared the light emission spectrum of paints in aqueous medium (PBS pH 7,4) in the absence and presence of HeLa cells. Without cells, DM has a wider and relatively flat absorption spectrum in the 400-500 nm excitation range, while AO has a sharp and twice as high excitation maximum, close to the laser line of 488 nm. The presence of cells did not change the absorption spectrum of dyes; However, the light scattering of the cells significantly dampened the intensity of the excitatory light. Remarkably, when the cells were excited at 480 nm, AO showed a maximum fluorescence emission of about 20 times higher than DM at around 530 nm. Compared to this peak, the DM emission maximum has shifted by about 40 nm to the red. The presence of cells did not significantly change the emission spectrum of dyes. From toxicity experiments, we determined that the LD50 toxicity value in human HeLa cells was 4 μM for both solutions with a difference

of about 10% (4.4×10^{-6} M for AO and 4.0×10^{-6} M for DM, at a confidence level of 95%, AO was between 3.4×10^{-6} M and 5.8×10^{-6} M, DM: 3.3×10^{-6} M and 4.8×10^{-6} M). Based on this, it can be stated that there is not much difference in toxicity between the two compounds, therefore diMICAAc may still be suitable for PDT therapeutic treatment.

AO has a known photosensitizing property; Therefore, we wanted to see if this property changes in the DM. The HeLa cells treated with photosensitives were illuminated with 470 nm led light, at a wavelength close to the absorption maximum of dyes, for an increasing period of time (0, 1, 3, 8 and 24 hours). We then tested the viability of the cells with an MTT test. Half of the cells treated with 2.5 μM DM or AO died for 42 and 76 minutes of continuous exposure to light, respectively, so DM triggered the destruction of HeLa cells earlier than AO. The LD50 flux of blue light illumination differed significantly for the two photosensitives: 11.3 J/cm^2 for DM and 22.2 J/cm^2 for AO. The 95% confidence levels were 9.7 and 12.5 J/cm^2 for DM and 18.3 and 22.0 J/cm^2 for AO. Based on this, it can be stated that DM is more phototoxic than in the case of blue LED lighting in continuous blue light.

It is known that AO is a suitable lysosome pH probe with red fluorescent emission, exciting green light. Consequently, we wanted to know the optical properties of the new compound. We conducted several microscopic studies and concluded that the two dyes look comparable to in human cells in the same 1-3 μM concentration range (15-30 minutes, at 37°C, medium). Confocal studies have shown that cells show no morphological signs of toxicity in these short-term applications (3-4 hours under physiological conditions). However, DM in the cytoplasm of HeLa cells, at the excitation of 405 nm laser light and in the blue fluorescence emission range, made lysosome-type vesicules more visible than in an AO 543 nm excitation and red emission range, and DM, in visual judgment, identified slightly smaller vesicular structures compared to AO.

At 488 nm excitation and green fluorescence emission range, AO showed much more effectively painted and brighter fluorescence than DM. At this wavelength of 488 nm, both paints best painted the nucleolus of living cells, probably ribosomal RNA. Interestingly, unlike AO, DM did not distinguish nuclear DNA from other cytoplasmic regions of cells. AO has been painting DNA in a constantly intensifying way, which is likely to be paralleled by the fact that illuminating light is increasingly stressing cells, causing increasing phototoxicity to the nucleus. This differentiated DNA painting is probably rooted in an an's greater fluorescence quantum efficiency and DM's smaller DNA binding. Using higher concentrations of paint, neither AO

nor DM could distinguish between lysosomes or nucleoluses, since both paints painted all components to a similar degree.

We performed confocal spectral imaging with two additional human and one mouse cell lines (SK-BR-3, OCM-1, NIH-3T3-G185) to see if other cells of tissue and race origin were painted and whether they had the same or different patterns of painting as human HeLa cells. Spectral imaging confirmed that all human and mouse cell types were painted with similar spectral patterns, and that the staining did not depend fundamentally on the origin of the cells. However, the overall intensity of cell painting was very different, which supports the idea that different membrane carriers and transporters are involved in the transport of paint for the two dyes. Mouse cells NIH-3T3-G185 are stable transfectants of the human MDR1 multidrug-transporter gene. These cells showed a higher intensity than DM, suggesting that AO is a better substrate of the human ABCB1 membrane transporter than DM. This property of DM is beneficial for lysosomal labeling.

Events of acute cell death, when the cell membrane ruptures, can be visualized; therefore, we decided to also monitor the dynamics of phototoxic processes using an iCys laser scanning cytometry imaging system. By measuring the flux at the sample level, we were able to conveniently control the lighting energy that reaches the cells by tracking the number of cycles used. To detect membrane ruptures when cells die, the medium containing photosensitisers was supplemented with a membrane impermeable red DNA dye, propidium iodide. After recording the entire process, we took wide-angle fluorescent images. However, at the end of the observational process, the scanned and consequently illuminated cells in the center of the image lost their green photosensitive paint due to the loss of membrane integrity, and the nuclei were painted red with DNA paint. In the transmission images, the contours of the cells were recognizable both in the center of the field of vision and on the periphery. The images have shown that irradiated light energy can be dosed locally and quantitatively, and that unlit cells provide inherently negative control.

We made a video of the cycle-making footage. We have seen the accumulation of photosensitives in cells, where AO, which has a higher quantum efficiency of fluorescence than DM, painted the cells more strongly. The lower quantum efficiency of DM was beneficial for better distinguishability of pi's appearance within cells. Initially, most of the cells were alive, and very few dead cells were painted red. First, cells attached to the bottom of the plate lose their membrane connections, pseudo-ododies and microvilluses. Then the cells shrink, lose membrane fragments and constantly strengthen membrane blebing, blistering develops.

Initially, the blisters were present in smaller numbers, then their number decreased and grew larger and larger. Eventually, some bleb grew to cell size, and suddenly most of the membrane structures retreated to the surface of the nuclei, and very few blebs remained stable. The blisters were initially transparent, then greenish in color. Suddenly, the greenish blebek turned reddish, while the nuclei of the cell and cytoplasm also changed color. Single-celled changes occurred in sync with a small scattering over time; consequently, photosensitives triggered cell changes under the influence of illumination.

Experiments to determine the kinetics of phototoxicity have shown that the kinetics of AO phototoxicity is faster fractionalized in 488 nm laser illumination. It is known that under physiological conditions chromatine remains in the nucleus unless, for some reason, the nucleus membrane is damaged and the nuclear DNA is fragmented to such a size that it can freely be released from the nucleus. It was obvious that large macromolecules remained in the nucleus, since eventually, within the entire sequence of images, red core residues were painted in dead cells with the highest intensity. Nevertheless, a huge amount of reddish-colored DNA escaped from the cells, possibly in a fragment that fell apart into smaller fragments, when DNA fragments were able to penetrate the phenesthetized nucleus and cytoplasm membranes damaged by light. We wanted to quantify the DNA lost from the cells using fluorescent images. However, there was one obstacle that did not make this goal possible. Propidium iodide does not paint well on the intrusive nuclei of living cells, even if the membrane inpermeable PI is directly transferred to the cytoplasm, since in the injury-free nucleus the DNA is tightly super-coiled. Similarly, this may be the reason why AO does not paint DNA well in living cells. Intact DNA must become loose so that paints can penetrate freely, which can be solved either by applying a "power" compound or by collecting a certain number of DNA strand splits. In the frames of single-cell kinetics, the green-red color change suggested that photo-damaged DNA is released from the nucleus, which can be made visible by the membrane-transient DNA dye, PI, when the cell membrane becomes permeable to PI due to photo damage. Therefore, the relative ink content of the images was quantified based on the intensity of the green and red colors. The images showed that DNA painting in the seeds became more intense. This proved that the loss of AO and DM and DNA labeling by PI clearly followed each other in time and showed only minor overlap. It is also interesting that the changes induced by AO preceded the changes induced by the DM.

The cells were illuminated with a flux of 0.9 J/cm^2 in each cycle during a 2.9 second panning of a cycle. For DM, 28 cycles were required for cell death and 24 cycles for AO, and these

exposure cycles corresponded to fluctuations of 26.3 J/cm^2 and 22.5 J/cm^2 respectively. This observation contrasted with the result of LED illumination, where DM was more deadly to cells. In addition to the difference between LSC laser and LED illumination, in addition to the difference in excitations of 488 and 470 nm, fractional versus were continuous illumination. After each irradiation period, for the LSC, the cells remained in the dark for about five minutes between the cyclic 488 nm laser illuminations. This allowed the cells to regenerate to a certain level after each irradiation cycle. The reversal of the killing effect of the two compounds between fractional and continuous illumination suggests that the primary cell targets of the compounds are different.

We evaluated the spatial and temporal patterns of pixel intensities within the cells and in the environment of the cells. The changes in the case of AO were much more pronounced and the green-red shift began earlier than in the case of DM. By analyzing the fluorescence quantitative data, we were able to notice a number of other exciting things. First of all, the green-red overlap period took a few cycles of time, which we indicated here in minutes. This means that both photosensitization loss and PI growth are slightly delayed within the cytoplasm in time, whatever mechanism is responsible for this. This delay should not be a light-scattering or delayed fluorescence phenomenon, as light scattering is completed within fractions of the femtosecond and fluorescence is completed within nanoseconds. It cannot be a simple delay due to dye diffusion, since the differences in concentration would be leveled out within seconds at the distances within these cells. Therefore, the most likely explanation is that these events reflect partially active balancing steps of the life signal remnants of dying cells with acute DNA and membrane damage. These steps to balance can include, for example, the binding of photosensitives to cytoplasmic or membrane proteins, or the transport of paints to or from confinement to vesicles. Secondly, AO kinetics suggests stronger DNA damage due to more intense and massive PI changes. The tangents and amplitudes of PI curves also show more robust changes for AO than for DM. The degree of fluorescence changes is not actually comparable in terms of DNA content, since the quantum degrees of an and DM differ significantly from each other. In the case of AO, all the red signals observed in nuclear, lysosomal and blebs are quickly saturated, unlike DM, where only the nuclear signal peaks. The red background signals in the surroundings of the cells indicate even more accurately the greater general release of DNA fragments from AO sensitized cells, which have amplitudes greater than the DM curves. Thirdly, the PI kinetic curves obtained for both photosensitives show the sudden release of DNA like shockwaves.

The intakt cells initially do not have a nuclear PI signal, but the blebeks are temporarily filled with the strong DNA signal of pi. This suggests that light and photosensitives cause acute DNA damage to the core, and that dna fragments that become smaller and looser diffuse and produce a bright PI signal in the cytoplasm as well. Later, this saturated intra-bleb DNA signal drops almost to the level of background signals, which makes it clear the transient nature of the DNA wave both in space and time. The distance dependence of the background signals can also be observed, which shows descending cascading amplitudes in farther places.

In previous publications, we have never seen such real-time observations of the release of DNA fragments from cells. DNA release from cells can be caused by DNA damage or, e.g. apoptotic conditions, or even locally, e.g. in the case of caspase activation. Experimental layouts like the ones we use can be good models for determining the mechanism of DNA release even in case of photo damage or other natural conditions closely related to it. Sudden DNA damage at the nucleus, which leads to the extracellular release of DNA fragments and their spread into the environment, may be exacerbated by the phenomenon observed by Pierzynska-Mach et al. It has been observed that an filled acidic lysosome migrates into the intrusions of the nucleus deep into the nucleus during programmed cell death.

We wondered what happens to histonoid proteins during light damage when DNA disappears from the nucleus. Recently, Prof. Dr. Gábor Szabó and his team developed a methodology called QUINESIn, which ultimately characterizes the superhelicity of genomic DNA by visually measuring the amount of histones and the nuclei of DNA in individual cells. From these measurements, we knew that with high salt treatment or the use of suitable intercalatory paints, histone H2A proteins are easily excreted from the nucleus compared to other histones. With a small modification, we were able to use this method to examine the photodynamic effect.

In short, at the beginning of the experiment, the cells were painted with a photosensitive and embedded in an agarose gel on an IBIDI slide similar to single-cell gel electrophoresis. The cells were then illuminated with a blue laser of the specified dose in the gel, maintaining physiological conditions, using the iCys imaging system. After that, the cell membranes were lysed in the agarose with a neutral pH, and then, using NaCl solutions with increasing concentrations, the histones were eliminated from chromatin. Finally, the histones left in the nuclei were painted with fluorescently marked antibodies and the cells in the IBIDI chamber were visualized and quantified using laser-scanning cytometry. NaCl solutions with increasing concentrations in different IBIDI chambers were used to proportionally remove histones. The technique resulted in sigmoidal histone rejection curves in normal chromatins. When we

illuminated the cells, we observed that the standard sigmoid shape of salt-induced histone excretion changed.

The measured changes indicated that histone cross-binding to chromatine, DNA fragmentation, dna loss from nuclei, and subsequent histone loss of cell nuclei occurred during both AO and DM sensitized photodynamic treatment, albeit at different levels. First, histone rejection by DNA intercalculation AO was more pronounced than in the case of DM. Secondly, in the case of AO, the significant histone loss was the combined effect of more severe fragmentation of DNA and subsequent DNA loss, histone rejection by DNA intercalation inks, and cross-binding of proteins by the same dye to DNA. Third, DM cross-linked H2A to DNA more and less fragmented DNA than AO. Furthermore, there was no phase-selective accumulation of photosensitisers in the cell cycle. Summing up the conclusions drawn from the QUINESIn experiments, in fractional light, AO damaged chromatin more significantly than DM.

It is known that AO also accumulates in living lysosomes, and in high concentrations it exhibits red fluorescence due to dimer formation. In blue light, AO also damages lysosomes, and it is less known that this is associated with a particularly spectacular fireworks-like flashing phenomenon. We wondered if the vesicles indicated by the DM and showing fluorescence by 405 nm excitation are lysosomes, and whether DM photo treatment damages lysosomes like AO.

We put together an experiment to visualize AO, DM, and Lysotracker Orange at the same time, while using the same spatial and temporal illumination. Our results showed that both AO and DM painted the lysosomes, and both paints caused photo damage. Before the use of controlled irradiation, all three fluorophores made similar vesicular structures visible within the cells. After localized illumination, fluorescence of an and DM paints disappeared in the cells, and Lysotracker Orange was unable to visualize the lysosomes even inside the AO or DM marked cells. However, the lysosomes in the unlit cells containing photosensitives were stained with Lysotracker Orange, which meant that the lysosomes remained intact without light. At the same time, light caused lysosome damage in both AO and DM.

Spectacular flashes that indicated damage to the lysosomes during the illumination of AO painted cells were also observed in DM painted cells. Although in DM painted cells, the phenomenon was less spectacular, since the quantum effect of the paint is much lower. A noticeable humpback was recognizable on the photobleaching curve seen in the cells treated with DM. This slight increase is similar to the large hump of the PHOTOBLEACHING curve

of AO, which is a consequence of dequenching fluorophores released from the methacromatic lysosome.

For this method, the time of the beginning of photodestruction is inversely proportional to the sensitivity of the membrane. Measuring the timing of photodestruction makes this technique suitable for cell biology of the stability of the lysosomes against peroxidation. The method has not been checked with other paints so far. Since our modified DM molecule showed similar lysosome destruction, this supports the interpretation of the effect of AO and confirms the applicability of the method. Based on this, it can be stated that both AO and DM paints caused lysosome permeabilation caused by light lighting.

To prove that DM also causes photo damage to lysosomes, we wanted to quantify the extent of the damage in a concentration-based way. Then the cells were constantly illuminated with blue light in a traditional plate reading experiment. Then the cells were painted with Lysotracker Orange dye, and the fluorescence intensity of the lysosomes was re-recorded. The results of the plate reader were inconclusive, but with the color camera of our LSC system, we took epifluorescent microscopic images of the samples (in the holes) and evaluated the images by cell-by-cell image analysis. The lysosomes were localized correlated with the concentration of paint, but the cells remained intact. This may have been the reason for the failure of traditional plate reader results. However, simple images of wells evaluated at the single cell level correctly identified the state of damage to the lysosomes. The cells not painted with the photosensitive contained dot-like lysoorange-painted dot lysosomes with a low cytoplasmic background. When lighting with a photosensitive was used, the number of dot-like lysosomes decreased, while cytoplasmic background fluorescence increased due to an increase in photosensitization concentration. LLOMe, a positive control of lysosome membrane breakdown, gave similar single-cell images as AO or DM. The average lysosome content of each cell was evaluated using fluorescence images of cells painted in Lysotracker Orange. In the treated cells, the average fluorescence intensity of the lysosomes was significantly lower than that of the control cells. We then inserted the dose response of lysosome inactivation in each cell, which showed that DM causes more pronounced lysosome damage. Based on this parameter, we calculated the semi-efficient dose of AO, which was 1.1 μM, while for DM it was 0.6 μM. The 95% confidence interval of the averages of LD50 values was 0.9 μM and 1.3 μM, respectively, 0.5 μM and 0.7 μM for AO and DM. The curve fit showed a statistically significant difference ($p=0.0003$). We also looked at additional imaging parameters, such as the average intensity of

cells, the standard deviation of the fluorescence intensity of cells, the entropy and energy of the image of the cells, all of which indicated the same trend.

These data suggested that DM damaged lysosomes more than AO. Thus, the polar isocyano group asymmetrically localized in the acridine ring can increase the lysosomal targeted effect of DM. Probably, the negative charge outwards increased the ability to attach to the lysosomes. It is also noteworthy that continuous illumination shifted the degree of cell damage compared to fractional illumination. Continuous irradiation increased the tendency of lysosome breakdown, while fractionation was dominated by DNA damage. These trends suggest that lysosomal membrane repair mechanisms may have more capacity than the mechanisms for repairing DNA damage. Proving this idea requires further experimentation.

6. Summary

Of the three newly synthesized acridine vase compounds, DM appeared to be the most promising cell marker and photosensitizing substance, as it had the most optimal optical properties, and on the other hand, it preserved the morphology of cells to the greatest extent and was the least toxic in the absence of light. When comparing AO and DM, it can be stated that the absorption maximum of both compounds is at 488 nm. Comparing emission spectra, DM has a wider emission spectrum. In terms of emission maximum, there is a 30 nm right shift in the case of DM compared to AO.

In living cells, DM signals lysosomes with intense green fluorescence when exciting 405 nm laser light, with a lower background fluorescence than AO. There is no detectable DNA or RNA signal in the optimal excitation and fluorescence emission range of DM, but the cytoplasmic marking of cells and retained morphology make it an effective cell marker.

In the case of continuous LED lamp illumination of 470 nm, DM had a higher phototoxicity than AO, while in the case of 488 nm fractional laser illumination, the phototoxicity trend was reversed; However, without light irradiation, there was no significant difference in the toxicity of the two compounds.

Comparing the cellular and molecular effects of 488 nm laser light lighting quantitatively, AO has been shown to be a more effective DNA pest, possibly due to the symmetry of AO, it may be more effective DNA pests, as it may have greater DNA binding. In the case of fractional irradiation of 488 nm, AO caused cell death sooner, first indicated by the formation of cell membrane blebs and then by the shock wave-like outflow of photo phrased core DNA. At the same time, DM showed greater efficacy in the photopermabilization of the lysosome membrane of HeLa cells. The difference between continuous and fractional photodynamic cell death and data on the cellular mechanisms behind it suggest that repairing lysosome damage in HeLa cells may have more capacity than repairing DNA damage.

DM signals lysosomes more specifically, probably because of its negative charge outwards, it can get into the lysosomes better and show a greater lysosome harmful effect, while AO is better integrated into nucleic acids and has a stronger DNA photophrasing properties around 488 nm. By increasing AO lysosome targeting, the photodestruction of the lysosomes can be amplified, which in its photodynamic effect can cause increased immunogenic cell death induction.

7. Publications

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Registry number: DEENK/292/2022.PL
Subject: PhD Publication List

Candidate: Csaba Bankó
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Bankó, C.**, Nagy, Z. L., Nagy, M., Szemán-Nagy, G., Rebenku, I., Imre, L., Tiba, A., Hajdu, A., Szöllősi, J., Kéki, S., Bacsó, Z.: Isocyanide Substitution in Acridine Orange Shifts DNA Damage-Mediated Phototoxicity to Permeabilization of the Lysosomal Membrane in Cancer Cells.
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   IF: 3.998

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IF: 21.522

Total IF of journals (all publications): 49,684
Total IF of journals (publications related to the dissertation): 10,637

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

31 May, 2022