Published in final edited form as:

Mitochondrion. 2008 June ; 8(3): 237–246. doi:10.1016/j.mito.2008.04.003.

Targeting of Mitochondria by 10-N-Alkyl Acridine Orange Analogues: Role of Alkyl Chain Length in Determining Cellular Uptake and Localization

Myriam E. Rodriguez¹, Kashif Azizuddin¹, Ping Zhang², Song-mao Chiu¹, Minh Lam⁴, Malcolm E. Kenney^{2,5}, Clemens Burda^{2,3,5}, and Nancy L. Oleinick^{1,5*}

¹Department of Radiation Oncology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, USA.

²Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106, USA.

³The Center for Chemical Dynamics and Nanomaterials Research, Case Western Reserve University, Cleveland, Ohio 44106, USA.

⁴Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106, USA.

⁵The Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio 44106, USA.

Abstract

10-N-nonyl acridine orange (NAO) is used as a mitochondrial probe because of its high affinity for cardiolipin (CL). Targeting of NAO may also depend on mitochondrial membrane potential. As the nonyl group has been considered essential for targeting, a systematic study of alkyl chain length was undertaken; three analogues (10-methyl-, 10-hexyl-, and 10-hexadecyl-acridine orange) were synthesized and their properties studied in phospholipid monolayers and breast cancer cells. The shortest and longest alkyl chains reduced targeting, whereas the hexyl group was superior to the nonyl group, allowing very clear and specific targeting to mitochondria at concentrations of 20–100 nM, where no evidence of toxicity was apparent. Additional studies in wild-type and cardiolipin-deficient yeast cells suggested that cellular binding was not absolutely dependent upon cardiolipin.

Keywords

10-N-alkyl acridine orange; cardiolipin; mitochondria; NAO analogues

1. Introduction

Mitochondria are highly abundant cytoplasmic organelles that play key roles in numerous biochemical processes, including TCA and urea cycles, oxidative phosphorylation, beta oxidation of fatty acids, cell calcium regulation, and signaling of apoptosis (Crompton, 1999). Mitochondria are also targets of certain drugs (Morgan and Oseroff, 2001), including

*To whom correspondence should be addressed: Nancy L. Oleinick, Ph.D., Department of Radiation Oncology, School of Medicine (BRB-324), Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4942, Phone: 216-368-1117, FAX: 216-368-1142, E-mail: nlo@case.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

a class of photosensitizing agents used in photodynamic therapy (PDT). PDT is an approved treatment for cancer and certain non-malignant conditions that uses a photosensitizing drug and visible light to generate singlet oxygen and other forms of reactive oxygen to exert cytotoxic effects on cells (Oleinick et al., 2002). Numerous reports have implicated mitochondria as the most sensitive intracellular site for PDT, critical for directly triggering apoptosis (Oleinick et al., 2002) (Morgan and Oseroff 2001) (Kessel and Luo, 1998)(Kessel et al. 1997).

Cardiolipin (CL), a phospholipid found uniquely in the inner membrane of mitochondria and at the contact sites between the inner and outer membranes, represents 13–15% of total mitochondrial phospholipids (Ardail, 1990) (Malisan and Testi, 2003). CL has important roles in certain pathologies, such as heart ischemia and Barth's syndrome, and factors such as diet and aging can change CL levels (McMillin and Dowhan, 2002). CL contains four unsaturated fatty acids per molecule and thus is easily attacked by singlet oxygen as well as by other oxidizing agents. For example, doxorubicin, which forms complexes with CL, is a potent chemotherapeutic agent inducing apoptosis (Schlame et al., 2000). In addition to its propensity for oxidation, CL is a potentially important target for PDT and other oxidative therapies, because it is associated with essential mitochondrial proteins, including cytochrome c and Bcl-2 family proteins. Oxidation of CL has been demonstrated following exposure of human breast cancer cells in culture to PDT sensitized by protoporphyrin IX (Kriska et al., 2005). CL forms both tight and loose associations with cytochrome c (McMillin and Dowhan, 2002) (Ott et al. 2002), and it has been proposed that upon oxidation, CL binds cytochrome c less tightly. The CL-bound form of cytochrome c is thought to initiate apoptosis *via* a lipid-transfer step involving mitochondrion-targeted Bid. A direct relationship between CL loss and cytochrome c release from the mitochondria has been identified as an initial step in the pathway to apoptosis (McMillin and Dowhan, 2002). In monolayer experiments, cytochrome c had a lower affinity for peroxidized CL (CL-OOH) than for native CL, but binding was restored when CL-OOH was reduced to CL-OH by phospholipid hydroperoxide glutathione peroxidase, the enzyme that directly reduces peroxidized lipids in cell membranes (Nomura 2000 et al.)(Kriska et al., 2005). It has also been proposed that the peroxidase activity of cytochrome c can catalytically oxidize CL (Kagan et al, 2005).

The fluorescent dye 10-N-nonyl-acridine orange (NAO) has been used as a highly specific probe of CL. In liposomes, the affinity of NAO for CL was 30 times greater than for other negatively charged phospholipids (phosphatidylserine and phosphatidyl inositol), and there was virtually no interaction of NAO with zwitterionic phospholipids (phosphatidyl choline and phosphatidyl ethanolamine) (Petit et al, 1992). The high-affinity binding of NAO to CL has been used to determine a number of properties of CL; *e.g.*, to image CL in cells by confocal microscopy (Jacobson et al, 2002), to measure mitochondrial mass per cell (Guidot, 1998), and to quantify the level of CL in the inner and outer leaflets of the mitochondrial inner membrane (Garcia Fernandez et al, 2002). NAO has also been used to reveal properties of the binding of photosensitizers in cultured cells. Wilson *et al.* (Wilson *et al.*, 1997) found that NAO competitively inhibited the uptake of Photofrin® into mitochondria, indicating that some photosensitizers might bind to CL of the inner mitochondrial membrane. Pc 4, a phthalocyanine photosensitizer first synthesized at Case Western Reserve University and now in clinical trial at University Hospitals Case Medical Center, was reported to be localized near CL based on fluorescence resonance energy transfer (FRET) from NAO to Pc 4 (Morris et al., 2003).

Questions have been raised regarding the factors important for specific mitochondrial uptake of NAO and binding to CL. Although early data suggested that the mitochondrial uptake of this dye did not depend on membrane potential (Petit, 1994), later studies reported the opposite (Jacobson et al, 2002). Initially, the high affinity of NAO for CL was thought to result from two essential interactions, the electrostatic interaction of the NAO quaternary ammonium with

the ionized phosphate residues of CL and hydrophobic interaction between adjacent chromophores (Petit et al., 1992). A more recent study found the most important factor for targeting of NAO to CL to be insertion of the nonyl chain into the bilayer at the hydrophobic surface created by the four fatty acid chains (Mileykovskaya et al., 2001). In order to more clearly evaluate the role of the 10-position of the acridine orange ring and the nonyl group, we synthesized three analogues of NAO bearing C-1-, C-6-, and C-16-alkyl chains in the 10 position. A variety of other derivatives of acridine orange have been made previously for different applications, e.g., for quantification and characterization of reticulocytes in whole blood (US Patent Numbers 5075556 and 5045433), as fluorescent probes for the cytochemical detection of cancer cells (Schwarz and Wittekind, 1982), for locating tumor cells possessing guanidinobenzoate (Steven et al., 1985), or for increasing sensitivity of cancer cells to chemotherapy (Valentini et al., 2006). Septinus *et al.* (Septinus *et al.*, 1983) investigated the thermodynamic and spectroscopic properties of a series of 10-N-alkyl derivatives of acridine orange with alkyl chains of 1 to 9 carbons in length. They found that the dimerization constant depended on the length of the alkyl residue and, not surprisingly, that NAO was the most hydrophobic dye of the series. To our knowledge, there has been no systematic study of how alkyl chain length affects both photophysical and cellular properties of 10-N-alkyl acridine orange derivatives. Here, we report a comparison of three NAO analogues with NAO in terms of (a) selectivity and affinity to CL in lipid monolayers, (b) uptake and localization into mitochondria of MCF-7c3 cells, and (c) some important photophysical properties. We find that all of the alkyl-acridine orange compounds show some selectivity for mitochondria and CL, with the hexyl derivative being the most active and specific. A comparison of wild-type and CL-deficient yeast cells suggests that binding is not absolutely dependent on CL.

2. Materials and methods

2.1. Chemicals

Acridine orange base, iodomethane, 1-bromohexane, 1-bromononane and 1-bromohexadecane were obtained from Aldrich, while benzene, toluene, DMSO, and chloroform were obtained from Fisher Scientific. RPMI 1640, fetal bovine serum, and penicillin/streptomycin were from Hyclone. Yeast extract was purchased from Difco Laboratories, peptone from Fischer Scientific and glucose from Sigma Chemical. Distilled water treated in a Millipore Milli-Q system was used.

2.2. Synthesis

3,6-Bis(dimethylamino)-10-methylacridinium Iodide (Acridine Orange 10-Methyl Iodide), MAO. The work of Yamagishi *et al.* served as a guide for this and the following syntheses (Yamagishi *et al.*, 1981). A mixture of 3,6-bis(dimethylamino)acridine (acridine orange base) (147 mg), CH₃I (953 mg) and benzene (8 mL) was heated (reflux) for 27 h and filtered. The solid was recrystallized from an ethanol–acetone solution (3:2, 50 mL), washed (ether), vacuum dried (40°C) and weighed (126 mg, 56%). UV-vis (CH₃OH) λ_{max} , nm (log ϵ): 495 (4.9). NMR (CDCl₃): δ 8.50 (s, 1H, 9-Ar H), 7.80 (d, 2H, 1,8-Ar H), 7.05 (d, 2H, 2,7-Ar H), 6.78 (s, 2H, 4,5-Ar H), 4.42 (s, 3H, N⁺CH₃), 3.38 (s, 12H, NCH₃). HRMS-FAB (m/z): [M - I]⁺ calcd for M as C₁₈H₂₂N₃I, 280.1814; found, 280.1816.

MAO is a brown solid. It is soluble in H₂O, CH₃OH, CH₂Cl₂ and dimethylformamide, and insoluble in toluene and hexanes.

3,6-Bis(dimethylamino)-10-hexylacridinium Bromide (Acridine Orange 10-Hexyl Bromide), HAO. A mixture of 3,6-bis(dimethylamino)acridine (124 mg), 1-bromohexane (782 mg) and CHCl₃ (5 mL) was heated (reflux) for 72 h and evaporated nearly to dryness by rotary evaporation (40 °C). The wet solid was chromatographed (basic Al₂O₃ III, CH₂Cl₂-ethanol,

80:1), washed (ether), vacuum dried (40 °C) and weighed (67 mg, 33%). UV-vis (CH₃OH) λ_{max} , nm (log ϵ): 495 (4.9). NMR (CDCl₃): δ 8.75 (s, 1H, 9-Ar H), 7.95 (d, 2H, 1,8-Ar H), 7.08 (d, 2H, 2,7-Ar H), 6.65 (s, 2H, 4,5-Ar H), 4.82 (t, 2H, N⁺CH₂), 3.35 (s, 12H, NCH₃), 1.98 (m, 2H, N⁺CH₂CH₂), 1.65 (m, 2H, N⁺(CH₂)₂CH₂), 1.40 (m, 4H, N⁺(CH₂)₃(CH₂)₂), 0.90 (t, 3H, N⁺(CH₂)₅CH₃). HRMS-FAB (m/z): [M - Br]⁺ calcd for M as C₂₃H₃₂N₃Br, 350.2596; found, 350.2584.

HAO is a brown solid. It is soluble in H₂O, CH₃OH, CH₂Cl₂ and dimethylformamide, and insoluble in toluene and hexanes.

3,6-Bis(dimethylamino)-10-nonylacridinium Bromide (Acridine Orange 10-Nonyl Bromide), NAO. A mixture of 3,6-bis(dimethylamino)acridine (69 mg), 1-bromononane (992 mg) and toluene (5 mL) was heated (reflux) for 4 h and evaporated nearly to dryness by rotary evaporation (40 °C). The wet solid was chromatographed (basic Al₂O₃ III, CH₂Cl₂-ethanol solution, 80:1), washed (ether), vacuum dried (40 °C) and weighed (81 mg, 66%). UV-vis (CH₃OH) λ_{max} , nm (log ϵ): 495 (5.1). NMR (CDCl₃): δ 8.80 (s, 1H, 9-Ar H), 7.96 (d, 2H, 1,8-Ar H), 7.05 (d, 2H, 2,7-Ar H), 6.62 (s, 2H, 4,5-Ar H), 4.83 (t, 2H, N⁺CH₂), 3.34 (s, 12H, NCH₃), 1.95 (m, 2H, N⁺CH₂CH₂), 1.62 (m, 2H, N⁺(CH₂)₂CH₂), 1.40 (m, 2H, N⁺(CH₂)₃CH₂), 1.26 (m, 8H, N⁺(CH₂)₄(CH₂)₄), 0.86 (t, 3H, N⁺(CH₂)₈CH₃).

NAO is a brown solid. It is soluble in CH₃OH, CH₂Cl₂ and dimethylformamide, slightly soluble in H₂O, and insoluble in toluene and hexanes.

3,6-Bis(dimethylamino)-10-hexadecylacridinium Bromide (Acridine Orange 10-Hexadecyl Bromide), HDAO. A mixture of 3,6-bis(dimethylamino)acridine (124 mg), 1-bromohexadecane (1420 mg) and CHCl₃ (5 mL) was heated (reflux) for 72 h and evaporated nearly to dryness by rotary evaporation (40 °C). The wet solid was chromatographed (basic Al₂O₃ III, CH₂Cl₂-ethanol solution, 80:1), washed (ether), vacuum dried (40 °C) and weighed (87 mg, 32%). UV-vis (CH₃OH) λ_{max} , nm (log ϵ): 495 (5.0). NMR (CDCl₃): δ 8.78 (s, 1H, 9-Ar H), 7.95 (d, 2H, 1,8-Ar H), 7.09 (d, 2H, 2,7-Ar H), 6.65 (s, 2H, 4,5-Ar H), 4.85 (t, 2H, N⁺CH₂), 3.35 (s, 12H, NCH₃), 1.98 (m, 2H, N⁺CH₂CH₂), 1.65 (m, 2H, N⁺(CH₂)₂CH₂), 1.42 (m, 2H, N⁺(CH₂)₃CH₂), 1.20 (m, 22H, N⁺(CH₂)₄(CH₂)₁₁), 0.86 (t, 3H, N⁺(CH₂)₁₅CH₃). HRMS-FAB (m/z): [M - Br]⁺ calcd for M as C₃₃H₅₂N₃Br, 490.4161; found, 490.4153.

HDAO is a brown solid. It is soluble in CH₃OH, CH₂Cl₂ and dimethylformamide, and insoluble in H₂O, toluene and hexanes.

2.2. Cell Culture

MCF-7c3 cells were obtained from Dr. C. Froehlich, Northwestern University. They were derived from human breast cancer MCF-7 cells by stable transfection of human CASP3 cDNA. The cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin in an atmosphere of 5% CO₂/95 % air at 37 °C in a humidified incubator. Cells were used for experiments when they were in exponential growth.

2.3. Yeast strain and growth conditions

CL-deficient FGY2(crd1 Δ) and the isogenic wild-type FGY3 *Saccharomyces cerevisiae* cells (Gohil, 2005) were obtained from Dr. Miriam Greenberg, Wayne State University, Detroit, MI. Yeast cells were grown to early stationary phase in YPD medium (1% yeast extract, 2% peptone, and 2% glucose).

2.3. Stock solutions

Stock solutions of NAO or NAO analogues were prepared in ethanol and stored in the dark at 4°C. They were diluted into water or growth medium for experiments.

2.4. Spectroscopy

NMR spectra and mass spectra were recorded with an INOVA 400 MHz spectrometer (Varian, Palo Alto, CA) and with a KRATOS MS25RFA instrument (Ion Tech, Manchester, UK), respectively. Absorption spectra were determined with a Varian Cary 50 Bio UV-vis spectrophotometer. Fluorescence spectra were monitored in a Varian Cary Eclipse spectrofluorometer. For FRET Studies MCF-7c3 cells were plated and processed as described for flow cytometry (see Section 2.6), except that cell pellets were resuspended in 2 mL of phenol red-free Hank's Balanced Salt Solution (HBSS) with 5 mM glucose. For estimation of FRET, cells loaded with Pc 4 as well as NAO or an analogue were excited at 488 nm, and the emission spectra were recorded between 498–800 nm.

2.6. Flow Cytometry

MCF-7c3 cells were plated in normal growth medium in 60-mm tissue culture dishes at 6×10^5 cells per dish, allowed to grow for 48 hr prior to addition of the desired dye for up to 60 min, and then harvested by trypsinization. Flow cytometric analysis was performed at the Case Comprehensive Cancer Center Flow Cytometry Core, using an EPICS Elite flow cytometer. NAO and its analogues were excited by a broadband UV laser (488 ± 5 nm), and fluorescence emission was collected with a 525 ± 5 nm band-pass filter. Autofluorescence of control cells was subtracted, and the mean value of the fluorescence was expressed relative to that of the corresponding average value for the same concentration of NAO as an indication of the relative amount of an analogue in the cells.

2.7. Confocal microscopy

Cells were plated in 35-mm glass-bottomed tissue-culture dishes (MatTek Corp., Ashland, MA) at 2×10^5 cells per dish. The cultures were incubated with specified concentrations of the dyes for 30 minutes. All confocal images were acquired using a 63× N.A 1.4 oil immersion planapochromat objective on a Zeiss LSM 510 confocal microscope in the Case Comprehensive Cancer Center Confocal Microscopy Core Facility. Images of all NAO analogues fluorescence were collected using a 488-nm excitation light from the argon laser, a 488-nm dichroic mirror, and a 500–550 nm band pass barrier filter. Controls without dye and with acridine orange were also run.

Co-localization of HAO with MitoTracker Deep Red (M-22426; Invitrogen) was determined by co-incubating 20 nM of HAO with 200 nM of Mitotracker Deep Red for 30 min. Imaging was done as above, except that a He/Ne2 laser supplied the 633-nm excitation light, a 633-nm dichroic mirror, and a 650-nm long pass filter were used to collect emission of Mitotracker Deep Red. The appropriate controls were run to insure that HAO excitation and emission were spectrally distinct from Mitotracker Deep Red excitation/emission.

2.8. Binding of NAO analogues to cardiolipin and other phospholipids

Binding of NAO analogues to CL was studied in lipid monolayers (Nomura, 2000) with slight modifications. Ninety-six-well microplates (Corning 3915) were coated with 50 μ L of 20 μ M CL solution in ethanol and evaporated at 37°C for 5 h. Increasing concentrations (0–120 μ M) of NAO or analogues were added in deionized water containing up to 1% ethanol. The plates were incubated with shaking for 30 min at room temperature protected from room light, then the wells were washed with 150 μ L deionized water three times, and residual dye was dissolved in 150 μ L DMSO with shaking at room temperature for 5 minutes. Finally, microplates were

read in a Victor ³V Wallac 1420 multilabel fluorescence plate reader (Perkin Elmer) using $\lambda_{\text{exc}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 535 \text{ nm}$. A standard curve was prepared using known concentrations of NAO or analogues in DMSO. To estimate selectivity of the NAO analogues to CL vs. other lipids, some wells were coated with 50 μL of a 20- μM ethanolic solution of dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE), or cholesterol (CHOL).

2.9 Binding of NAO and HAO to wild type and cardiolipin-deficient yeast cells

Quantification of CL using NAO or HAO was performed as described Gohil *et al.* (Gohil, 2005) with slight modifications. Cells were fixed in cold ethanol (70%), washed three times with cold buffer (10 mM Tris/HCl, pH 7.0), vortexed vigorously, sonicated to eliminate aggregates, and counted using a hemocytometer. Yeast cells (2 or 5×10^7) were incubated with 45 μM NAO or HAO for 15 min at 20°C. Cells were washed three times in buffer to remove unbound dye and resuspended in 1.5 mL of buffer. Fluorescence Intensity was measured at 640 nm upon excitation at 450 nm.

3. Results

3.1. Synthesis

The synthesis of the acridine orange 10-alkyl halides is summarized in Scheme 1, and the structures, synthetic yield data, and solubility data are summarized in Table 1. Not surprisingly, the synthesis of the long chain halides proceeds more slowly than the short chain ones. The syntheses probably would proceed more rapidly and with greater yields if a higher boiling solvent such as toluene were used.

3.2. Spectroscopic properties of the NAO analogues

NAO analogues were characterized by their UV-Vis spectra and fluorescence spectra. For all four compounds, the absorption maximum was at 495 nm in methanol, and extinction coefficients were similar to that of NAO ($84,000 \text{ cm}^{-1} \text{ M}^{-1}$). Upon excitation at 488 nm, the emission spectra were also similar between 498–800 nm. For each concentration, the same area under the fluorescence curve was found for the four compounds, which indicates similar fluorescence quantum yields for all the dyes (data shown in Fig. 1 for HDAO as example). Since the chromophore was not changed by the addition of the variously sized alkyl chains at the 10-N position, the similarity in spectral properties was expected.

3.3. Cellular uptake of NAO and analogues evaluated by flow cytometry

Because the four dyes have the same spectroscopic properties, it is possible to compare cell uptake using fluorescence. Cell cultures were exposed to 100 or 250 nM of each dye for 30 min, after which the cells were collected and analyzed by flow cytometry. Figure 2a shows that the length of the 10-N alkyl chain has a marked influence on the ability of the four compounds to enter MCF-7c3 cells. MAO and NAO have similar uptake while less than one-third as much HDAO enters the cells as does NAO. Unexpectedly, HAO showed the highest uptake, with values 2–3 times greater than those for NAO.

Because the differences found in uptake after a 30-min incubation could reflect different rates of uptake that might result in equal levels of all the dyes if sufficient time is allotted, we compared the uptake of HAO and NAO (250 or 100 nM) into MCF-7c3 cells after 10, 30 or 60 min of incubation. Figure 2b shows that both HAO and NAO enter the cells quickly, reaching maximum values by 30 min, with little or no further uptake at 60 min. At all times and at both concentrations, more HAO than NAO entered the cells. Additional control experiments showed that in phenol red-free HBSS at three different concentrations (150, 250 and 500 nM), the shape

and intensity of the emission spectra were similar for HAO and NAO at equal concentration. Thus, uptake is effectively different in MCF-7c3 cells.

3.4. Localization of MAO, HAO, NAO and HDAO assessed by confocal microscopy

NAO has been shown to bind selectively to mitochondria as a result of the mitochondrial membrane potential and/ or CL content. We next compared the selectivity of the three analogues for binding to mitochondria using confocal microscopy. Concentrations of each dye were chosen to yield approximately equal amounts in the cells, based on the uptake data of Fig. 2a. Figure 3a shows that at the lower concentration, MAO, HAO, and NAO show excellent specificity for mitochondria. In contrast, HDAO appears to bind to mitochondria but also diffusely in the cytoplasm. At the 2.5-times-higher concentrations, none of the compounds binds selectively to mitochondria, and all show evidence of toxicity, e.g., presence of vacuoles.

HAO was also tested at lower concentrations; fluorescence images indicating excellent selectivity for mitochondria could be observed at concentrations of HAO as low as 20 nM (Fig. 3b). Confocal microscopy also revealed nearly complete colocalization of HAO with a mitochondrion-specific probe (Mitotracker Deep Red), confirming that HAO at the low concentration binds specifically to mitochondria (Fig. 3c).

3.5. Binding of NAO and analogues to lipid monolayers

3.5.1. Binding of NAO analogues to cardiolipin—Evidence has accumulated for specific binding of NAO to CL in subcellular systems, including lipid monolayers (Nomura, 2000) and liposomes (Petit, 1994). We chose to study binding of increasing amounts of each NAO analogue to monolayers formed from 1 nmole of CL in each well of a 96-well plate. As shown in Figure 4a, the titration curves were similar for MAO, HAO, and NAO; *i.e.*, they presented the same fluorescence intensity in DMSO, with HDAO showing more variable fluorescence values. These data suggest that the intrinsic affinities of the four dyes for CL are similar, and that other factors must explain their different abilities to enter cells and bind to mitochondria.

This experiment was repeated but coating the microplate with a higher level (2.5 nmoles) of CL or with a combination of 1 nmole each of CL and DMPC (data not shown). The shape of the binding curves and the saturation values were similar, indicating that the affinity of the dyes for CL was not affected.

For all of the dyes, a plateau in the fluorescence intensity was reached at concentrations above 50 μ M; this maximum value was similar for the four compounds. Some irregularities were observed for HDAO at concentrations below 50 μ M; similar irregularities were observed in background values, *i.e.*, binding of HDAO to empty wells. Perhaps the long hexadecyl chain interferes with the interaction between CL and HDAO at low concentrations, whereas the presence of more molecules of HDAO may drive the interaction. If true, this property could explain the poor uptake of HDAO into cells, as judged by flow cytometry, as well as the relatively poor specificity for mitochondria, as judged by confocal microscopy.

3.5.2. Selectivity of NAO analogues to cardiolipin—In order to evaluate how selective the NAO analogues are for binding to CL, other lipid monolayers were compared. For these experiments, wells were coated with equal amounts of DMPC, DMPE, CL or CHOL, and the extent of binding of NAO or analogues was studied. The binding data were normalized to the amount of each dye bound to CL. As shown in Fig. 4b, MAO, HAO and NAO bound preferentially to CL; binding to DMPC or DMPE and to CHOL was less than 10% and 35% of that to CL, respectively. The background binding to wells without lipid was often in the range of up to 5% of the CL binding, indicating that there was little or no specific binding to

lipids other than CL. In contrast, HDAO showed less selectivity to CL than did the other dyes. HDAO binding to CHOL ranged from 50–200% of its binding CL; its binding to DMPE varied from below background to almost 40% of its binding to CL; and it showed the highest binding to empty wells. These data for HDAO indicate that the hexadecyl chain imparts high hydrophobicity to the molecule and must be an obstacle to specific binding. The combined data show that the length of the alkyl chain is important for stabilizing the interaction with CL.

3.6. FRET studies

With the goal of studying the initial PDT-induced events causing cell death, we provided evidence for colocalization of the photosensitizer Pc 4 and CL based on the ability of NAO to transfer resonance energy to Pc 4 within cultured cells (Morris *et al.*, 2003). We now confirm FRET from NAO (300 or 500 nM) to Pc 4 (200 nM) and moreover extend the finding to include FRET from HAO (150 or 250 nM) to Pc 4 (200 nM). As shown in Fig. 5, approximately equal levels of FRET to Pc 4 were observed for 150 nM HAO and 300 nM NAO, concentrations that produce approximately equal levels of these two dyes in the cells. A larger FRET peak was observed at the higher HAO and NAO levels (data not shown).

3.7 Binding of NAO and HAO to wild type and cardiolipin-deficient yeast cells

The above results suggest that at least some of the specificity of NAO and HAO for mitochondria could be due to their preferential binding to CL. Whether or not that specificity is absolute was tested by comparing the binding of the alkyl acridine orange derivatives to yeast cells that were either wild-type or genetically deficient in CL. We followed the procedure described by Gohil *et al* (Gohil, 2005), who found no appreciable difference between the two cell types in the binding of NAO (45 μ M). Using the same conditions and concentration, we confirmed that both cell types bind the same amount of NAO (Fig. 6a). A similar absence of difference between the two cell types was obtained with 45 μ M HAO (Fig. 6b). Moreover, there was no difference between the two cell types when they were compared using 2×10^7 or 5×10^7 cells of each type.

4. Discussion

Our systematic study of the role of alkyl chain length on properties of 10-N-alkyl acridine orange derivatives has revealed that all of the compounds have essentially identical spectroscopic properties, as expected because no change was made to the acridine orange chromophore. However, important differences among the four compounds were found. (1) A long alkyl group at position 10 is not absolutely necessary for binding to a lipid monolayer formed by CL, since all derivatives demonstrated similar affinity for CL. (2) Nevertheless, MAO, HAO, and NAO have much greater affinity for CL than for DMPC, DMPE, or CHOL. HDAO, because of its very long alkyl chain, demonstrates considerable binding to CHOL. (3) The alkyl chain plays an important role in the cellular uptake of the compounds. Of the four dyes, HAO demonstrated the most efficient uptake into MCF-7c3 cells and specific mitochondrial fluorescence at low concentrations. (4) All of the dyes except HDAO revealed specificity for mitochondria at sufficiently low concentrations but diffuse localization within the cells at higher concentrations that also produced evidence of toxicity. This last finding is in agreement with the results of Maftah *et al.* (Maftah *et al.*, 1990), who showed that high concentrations (3–10 μ M) of NAO interfered with state-3 respiration and ATP synthesis but also other steps in oxidative phosphorylation. (5) When studied at concentrations producing similar uptake and mitochondrial binding, HAO and NAO produced similar levels of FRET to Pc 4. (6) Confirming and extending earlier results with yeast (Jacobson, 2002) (Gohil, 2005), we find that neither NAO nor HAO showed any preferential binding to wild-type as compared to CL-deficient yeast. Thus, it appears that the preference for binding to CL is not absolute. Gohil *et al.*, from whom the cells were obtained, found three main CL species in wild-

type extracts, but none of these species were found in CL-deficient cells. Therefore, the remaining CL species could be responsible for these results; alternatively, in the absence of CL, the relatively hydrophobic NAO and HAO may bind to other lipids. Neither reagent should then be used to quantify CL in cells.

Mileykovskaya *et al.* (Mileykovskaya, 2001) proposed a model for the binding of NAO to CL in which the nonyl group plays a crucial role. It is difficult to know how relevant that model is to the binding of the alkyl acridine dyes to cells, because their study employed very concentrated methanolic solutions of NAO to determine the fluorescence spectral shift as the NAO concentration is raised. Based on those spectral shifts, the model was developed to explain how CL may concentrate NAO at its surface. On the one hand, the concentrations of NAO employed in those studies were many orders of magnitude higher than those in our cellular studies, and the interpretation of the spectral measurements did not consider possible self-absorption and reemission of the sample at those concentrations. On the other hand, we would still agree that the alkyl chain is important in the binding of alkyl acridine dyes to cells, as supported by our data in Figure 2 and Figure 3. However, even MAO, with the shortest possible alkyl chain, bound to mitochondria specifically but with lower efficiency than HAO or NAO. These data show that a long alkyl chain is not essential for cellular binding and provide evidence for a crucial role of the cation to interact with the phospholipid anion.

In conclusion, HAO appears to be a superior acridine orange derivative for imaging mitochondria, because it is useful at very low concentrations with minimal perturbation of the cells.

Acknowledgements

This research was supported by grants from the US National Cancer Institute, DHHS: R01 CA106491 and P30 CA43703. The authors thank Dr. Miriam Greenberg and her student, Amit Joshi, Wayne State University, Detroit, for the wild type and mutant yeast cells and Dr. Steven Sanders, Case Western Reserve University, Cleveland, OH, for assistance in culture of the yeast cells.

References

- Ardail D, Privat JP, Egret-Charlier M, Levrat C, Lerme F, Louisot P. Mitochondrial contact sites. Lipid composition and dynamics. *J. Biol. Chem* 1990;265:18797–18802. [PubMed: 2172233]
- Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem. J* 1999;341:233–249. [PubMed: 10393078]
- Garcia Fernandez M, Troiano L, Moretti L, Nasi M, Pinti M, Salvioli S, Dobrucki J, Cossarizza A. Early changes in intramitochondrial cardiolipin distribution during apoptosis. *Cell Growth Differ* 2002;13:449–455. [PubMed: 12354754]
- Gohil V, Gvozdenovic-Jeremic J, Schlame M, Greenberg M. Binding of 10-N-nonyl acridine orange to cardiolipin-deficient yeast cells: implications for assay of cardiolipin. *Anal Biochem* 2005;343:350–352. [PubMed: 15963941]
- Guidot DM. Endotoxin pretreatment in vivo increases the mitochondrial respiratory capacity in rat hepatocytes. *Arch. Biochem. Biophys* 1998;354:9–17. [PubMed: 9633592]
- Jacobson J, Duchon MR, Heales SJ. Intracellular distribution of the fluorescent dye nonyl acridine orange responds to the mitochondrial membrane potential: implications for assays of cardiolipin and mitochondrial mass. *J. Neurochem* 2002;82:224–233. [PubMed: 12124423]
- Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritov VB, Amoscato AA, Osipov AN, Belikova NA, Kapralov AA, Kini V, Vlasova II, Zhao Q, Zou M, Di P, Svistunenko DA, Kurnikov IV, Borisenko GG. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat. Chem. Biol* 2005;1:223–232. [PubMed: 16408039]
- Kessel D, Luo Y. Mitochondrial photodamage and PDT-induced apoptosis. *J. Photochem. Photobiol. B* 1998;42:89–95. [PubMed: 9540214]

- Kessel D, Luo Y, Deng Y, Chang CK. The role of subcellular localization in initiation of apoptosis by photodynamic therapy. *Photochem. Photobiol* 1997;65:422–426. [PubMed: 9077123]
- Kriska T, Korytowski W, Girotti AW. Role of mitochondrial cardiolipin peroxidation in apoptotic photokilling of 5-aminolevulinate-treated tumor cells. *Arch. Biochem. Biophys* 2005;433:435–446. [PubMed: 15581600]
- Maftah A, Petit JM, Julien R. Specific interaction of the new fluorescent dye 10-N-nonyl acridine orange with inner mitochondrial membrane. A lipid-mediated inhibition of oxidative phosphorylation. *FEBS Lett* 1990;260:236–240. [PubMed: 2298301]
- Malisan F, Testi R. Mitochondrial lipids as apoptosis regulators. *Curr. Med. Chem* 2003;10:1573–1580. [PubMed: 12871128]
- McMillin JB, Dowhan W. Cardiolipin and apoptosis. *Biochim. Biophys. Acta* 2002;1585:97–107. [PubMed: 12531542]
- Mileykovskaya E, Dowhan W, Birke RL, Zheng D, Lutterodt L, Haines TH. Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces. *FEBS Lett* 2001;507:187–190. [PubMed: 11684095]
- Morgan J, Oseroff AR. Mitochondria-based photodynamic anti-cancer therapy. *Adv. Drug. Deliv. Rev* 2001;49:71–86. [PubMed: 11377804]
- Morris RL, Azizuddin K, Lam M, Berlin J, Nieminen AL, Kenney ME, Samia AC, Burda C, Oleinick NL. Fluorescence resonance energy transfer reveals a binding site of a photosensitizer for photodynamic therapy. *Cancer Res* 2003;63:5194–5197. [PubMed: 14500343]
- Nomura K, Imai H, Koumura T, Kobayashi T, Nakagawa Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis. *Biochem. J* 2000;351:183–193. [PubMed: 10998361]
- Oleinick NL, Morris RL, Belichenko I. The role of apoptosis in response to photodynamic therapy: what, where, why, and how. *Photochem. Photobiol. Sci* 2002;1:1–21. [PubMed: 12659143]
- Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. *Proc. Natl. Acad. Sci. USA* 2002;99:1259–1263. [PubMed: 11818574]
- Petit JM, Maftah A, Ratinaud MH, Julien R. 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipids in isolated mitochondria. *Eur. J. Biochem* 1992;209:267–273. [PubMed: 1396703]
- Schlame M, Rua D, Greenberg ML. The biosynthesis and functional role of cardiolipin. *Prog. Lipid. Res* 39:257–288. [PubMed: 10799718]
- Schwarz G, Wittekind D. Selected aminoacridines as fluorescent probes in cytochemistry in general and in the detection of cancer cells in particular. *Anal Quant. Cytol* 1982;4:44–54. [PubMed: 6176157]
- Septinus M, Seiffert W, Zimmermann HW. Hydrophobic acridine dyes for fluorescence staining of mitochondria in living cells. Thermodynamic and spectroscopic properties of 10-N-alkylacridine orange chlorides. *Histochemistry* 1983;79:443–456. [PubMed: 6197394]
- Steven FS, Griffin MM, Al-Ahmad RK. The design of fluorescent probes which bind to the active centre of guanidinobenzoate. Application to the location of cells possessing this enzyme. *Eur. J. Biochem* 1985;149:35–40. [PubMed: 2581779]
- Valentini A, Pucci D, Crispini A, Federici G, Bernardini S. Acridine Orange based platinum(II) complexes inducing cytotoxicity and cell cycle perturbation in spite of GSTP1 up-regulation. *Chem. Biol. Interact* 161:241–250. [PubMed: 16814760]
- Wilson BC, Olivo M, Singh G. Subcellular localization of Photofrin and aminolevulinic acid and photodynamic cross-resistance in vitro in radiation-induced fibrosarcoma cells sensitive or resistant to photofrin-mediated photodynamic therapy. *Photochem. Photobiol* 1997;65:166–176. [PubMed: 9066298]
- Yamagishi A, Masui T, Watanabe F. Selective activation of reactant molecules by reversed micelles. *J. Phys. Chem* 1981;85:281–285.

Fig. 1. Absorption (dashed line) and fluorescence (solid line) spectra for HDAO (4 μ M) in methanol.

Fig. 2.

Uptake of MAO, HAO, NAO, and HDAO into MCF-7c3 cells. **a)** Cells were exposed to 100 nM or 250 nM of each dye for 30 min at 37 °C, then analyzed for cell-bound fluorescence by flow cytometry. *Arbitrary value of 1 was assigned to the average fluorescence value for 100 nM NAO. Results are the average of duplicate determinations in a single experiment which was repeated once with similar results. **b)** Cells were exposed to the same concentrations of the dyes as for panel **(a)** but for different incubation times. Data represent individual (circles) and average (bars) values of duplicates from one experiment. This experiment was repeated twice more with similar results.

Fig. 3. Subcellular localization of MAO, HAO, NAO, and HDAO, as revealed by confocal microscopy. **a)** Cells were incubated with the indicated concentrations of the dyes for 30 min before images were recorded. **b)** Cells were incubated with 20 nM HAO **c)** Cells were incubated with 20 nM HAO and 200 nM Mitotracker Deep Red for 30 min.

Fig. 4.

Binding of NAO analogues to lipid monolayers. **a)** Increasing amounts of each dye were added to lipid monolayers in 96-well plates, as described in Methods. After washing, the retained dye was dissolved in DMSO and its fluorescence measured ($\lambda_{\text{excitation}} = 485 \text{ nm}$; $\lambda_{\text{emission}} = 535 \text{ nm}$). Data are presented as the average of duplicate values at each concentration of the respective dye. Data from a single experiment are shown; this experiment was repeated twice more with similar results. **b)** Selectivity of binding of NAO analogues to lipid monolayers. Monolayers were formed from 1 nmole of each lipid (DMPC, DMPE, CL, and CHOL). Results from one representative experiment are shown; each bar represents an average of data from 4 wells $\pm$ standard deviation. Two additional repeat experiments gave comparable results.

Fig. 5. FRET from NAO or HAO to Pc 4. The solid line represents fluorescence of MCF-7c3 cells alone and MCF-7c3 cells with 200 nM Pc 4; dotted lines represent fluorescence of cells with Pc 4 plus 150 nM HAO, and dashed lines represent fluorescence of cells with Pc 4 plus 300 nM NAO. The inset provides a magnified view of the long wavelength portion of the main graph.

Fig. 6.

3.7 Binding of NAO and HAO to wild type and cardiolipin-deficient yeast cells. Yeast cells (2 or 5×10^7) prepared as described in Materials and Methods, were incubated with $45 \mu\text{M}$ NAO (panel A) or HAO (panel B) 15 min at 20°C . Cells were washed three times in buffer to remove unbound dye and resuspended in 1.5 mL of buffer for determination of fluorescence (λ_{exc} , 450 nm; λ_{em} , 640 nm).

type	X	n	yield (%)
MAO	I	1	56
HAO	Br	6	33
NAO	Br	9	66
HDAO	Br	16	32

Scheme 1.
Synthesis of acridine orange 10-alkyl halides

Table 1
Structure and solubility data for acridine orange 10-alkyl halides

	structure	H ₂ O	CH ₃ OH	CH ₂ Cl ₂	DMF ^a	C ₈ H ₅ CH ₃	C ₆ H ₁₄
MAO		^b s	s	s	s	i	i
HAO		s	s	s	s	i	i
NAO		ss	s	s	s	i	i
HDAO		i	s	s	s	i	i

^aDMF, dimethylformamide.
^b s, soluble; ss, slightly soluble; i, insoluble.