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Real-time Visualization of Photochemically Induced Fluorescence of 8-Halogenated Quinolones: Lomefloxacin, Clinafloxacin and Bay3118 in Live Human HaCaT Keratinocytes[†]

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Abstract

Halogenoquinolones are potent and widely used antimicrobials blocking microbial DNA synthesis. However, they induce adverse photoresponses through the absorption of UV light, including phototoxicity and photocarcinogenicity. The phototoxic responses may be the result of photosensitization of singlet oxygen, production of free radicals and/or other reactive species resulting from photodehalogenation. Here, we report the use of laser scanning confocal microscopy to detect and to follow the fluorescence changes of one monohalogenated and three di-halogenated quinolones in live human epidermal keratinocyte cells during *in situ* irradiation by confocal laser in real time. Fluorescence image analysis and co-staining with the LysoTracker probe showed that lysosomes are a preferential site of drug localization and phototransformations. As the lysosomal environment is relatively acidic, we also determined how low pH may affect the dehalogenation and concomitant fluorescence. With continued UV irradiation, fluorescence increased in the photoproducts from BAY y3118 and clinafloxacin, whereas it decreased for lomefloxacin and moxifloxacin. Our images not only help to localize these phototoxic agents in the cell, but also provide means for dynamic monitoring of their phototransformations in the cellular environment.

INTRODUCTION

Fluoroquinolones (FLQs) are antimicrobial therapeutic agents used in the treatment of many infectious diseases, as a consequence of their gram-negative bacterial activity (inhibition of DNA gyrase), as well as their gram-positive bacterial activity (inhibition of DNA type-IV topoisomerase) (1–5). The primary mode of bactericidal action of this class of drugs involves inhibiting DNA synthesis by cleaving bacterial DNA from the DNA-enzyme complexes formed with DNA gyrase and type-IV topoisomerase, thereby resulting in rapid bacterial death (6,7). All of these quinolone drugs absorb in both the UVB and UVA regions and therefore may induce dermal phototoxic reactions, including phototoxicity, photoallergy, photomutagenicity and photocarcinogenicity in animals and humans (8).

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The phototoxic effects induced by FLQs upon interaction with UV photons have been generally attributed to the generation of free radicals, and reactive oxygen species, such as superoxide anion ($\cdot\text{O}_2^-$), singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\text{OH}^\cdot$) (9–11). Although there is no direct correlation between the generation of singlet oxygen and the order of phototoxicity, there is some correlation between superoxide production and phototoxicity (9). In addition, there have been reports that these compounds produce stable toxic photoproducts which could lead to *in vivo* phototoxicity (12).

Due to their amphoteric nature, the photochemical behavior of FLQs strongly depends on the medium and the pH of the dissolving solvent (13,14). Under neutral conditions, where the zwitterionic forms of FLQs predominate, UVA absorption causes heterolytic defluorination at the C-8 position as the major photochemical pathway, and highly reactive intermediates, such as aryl cations or carbenes are formed (9,10,15,16). However, the charge distribution may change depending on where in the cell the antibiotic accumulates.

Here, we have used laser scanning confocal microscopy to examine both the subcellular localization and time course of photosensitization for three selected di-halogenated quinolones (Fig. 1) in human keratinocyte (HaCaT) cells, along with one monohalogenated quinolone for comparison (Fig. 1). We have used fluorescence image analysis and co-staining with the LysoTracker probe (Molecular Probes, Carlsbad, CA) to reveal that these four compounds are preferentially localized in the lysosomes. As the lysosomal environment is relatively acidic (17), we have also examined the fluorescence under acidic conditions. These results provide a unique real-time, spatially resolved visualization of photosensitization in a cellular environment induced by known reactions of photoactive drugs.

MATERIALS AND METHODS

Chemicals

Lomefloxacin was purchased from Sigma/Aldrich Chemical Co. (St. Louis, MO). BAY y3118 (8-chloro-1-cyclopropyl-6-fluoro-7-(octahydropyrido[3,4-*c*]pyridine-2-yl)-4-oxo-1,4-dihydroquinidine-3-carboxylic acid) and moxifloxacin (BAY8039) were provided by Bayer AG. Clinafloxacin was supplied by Parke-Davis Pharmaceutical Research (Ann-Arbor, MI). All were used without further purification. The 10 mM and 50 mM phosphate buffers at pH 7.4 were prepared using 0.1 M potassium dihydrogen phosphate and 0.1 M sodium hydroxide, chemicals supplied by Aldrich Chemical Co. (Milwaukee, WI). Absolute ethanol and absolute methanol were obtained from J.T. Baker (Phillipsburg, NJ). Quinine sulfate in 1N H_2SO_4 , used as reference in the fluorescence quantum yield measurements, was supplied by Aldrich. Glacial acetic acid, reagent grade sodium chloride, sodium fluoride and sodium hydroxide, used in the preparation of the total ionic strength adjustor for the calibration of the fluoride ion electrode in the defluorination studies, were also obtained from Aldrich.

Absorption and emission studies

Absorption studies were conducted using an HP diode-array 8451 spectrophotometer (Hewlett Packard Co., Palo Alto, CA). Emission spectra were recorded on a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ) with FluorEssence, having Windows compatibility. Slits were set to 2 nm. The emission spectra were corrected for photomultiplier tube monochromator response using built-in correction factors. The fluorescence spectra were also normalized for absorption at the excitation wavelength ($\lambda_{\text{exc}} = 350 \text{ nm}$) in accordance with the Beer–Lambert law.

Laser scanning confocal microscopy

The human keratinocyte (HaCaT) cells were seeded in 35 mm dishes containing a glass cover-slip-covered 15 mm cutout (matTek, Ashland, MA) for live cell microscopy measurements. The following day, the cells were incubated with solutions of moxifloxacin (20 μ M), lomefloxacin (20 μ M), clinafloxacin (60 μ M) and BAYy3118 (20 μ M) in PBS-CMF containing 10 mM glucose at 37°C for 1 h. Cells were located using visible light and then exposed to UVA irradiation (364 nm) at the time of excitation and detection. The fluorescence generated in the cells was monitored as a function of time using a Zeiss 510 Meta confocal microscope (Thornwood, NY) with excitation at 364 nm.

RESULTS

All four of the quinolones studied exhibited two absorption bands in the UV (Fig. 2). The more intense absorption maxima are in the 265–315 nm region (UVB), with the weaker absorption bands centered around 320–360 nm (UVA). All four also fluoresced in the visible at physiological pH (Fig. 3): each exhibited a broad band with a maximum between 425 and 475 nm. Continued irradiation resulted in a marked increase in fluorescence for clinafloxacin and Bay3118 and a rapid increase in quantum yield for lomefloxacin (data not shown).

We used laser scanning confocal microscopy to examine the subcellular localization of photosensitization in human keratinocyte (HaCaT) cells. Fluorescence image analysis and co-staining with the LysoTracker probe revealed that lysosomes are a preferential site of localization and phototransformation of these halogenoquinolones (Fig 4).

As the lysosomal environment is relatively acidic (~pH 4.8), we checked the fluorescence at pH 4 for each of the quinolones (Fig. 5), and each continued to fluoresce at the lower pH. Lomefloxacin and clinafloxacin showed a small redshift (~15 and 11 nm, respectively) and decreased in intensity to about half, whereas moxifloxacin showed the same intensity decrease with a larger redshift of ~43 nm. Bay 3118 fluorescence increased at the lower pH, with a redshift of ~18 nm. Thus, all of these quinolones continue to fluoresce while they are within the lysosomes, providing a convenient way to monitor them within cells.

Also with laser scanning confocal microscopy, we examined the time course of quinolone fluorescence in human keratinocyte (HaCaT) cells under UV irradiation. In the temporal fluorescence profiles (Fig. 6), the fluorescence of clinafloxacin and BAYy3118 (both with a Cl-atom at the eighth position) increased quickly. Moxifloxacin, which has a methoxy group rather than a halogen at the eighth position, showed an intense initial fluorescence that decreased somewhat upon prolonged irradiation in the cellular environment.

DISCUSSION

All four of these quinolones absorb in the UV region and are therefore potentially phototoxic. Upon absorption of a UVA or UVB photon, the FQ is transformed from its ground state (^1FQ) into an excited singlet state ($^1\text{FQ}^*$). From this state, the FQ may decay directly back to the ground state by emitting fluorescence which can be used for photodetection purposes. Alternatively, the FQ may undergo electron spin conversion to its triplet state ($^3\text{FQ}^*$), react with oxygen to form singlet oxygen or superoxide or fragment to form radicals or reactive intermediates.

In general, there are mainly five ways in which the excited states of FQs can photofragment:

1. Dehalogenation (photodefluorination or photodechlorination) at the eighth position of the quinolone moiety as fluoride and/or chloride ion.

2. Defluorination at the sixth position of the quinolone moiety induced by an electron-transfer procedure.
3. Dealkylation or group modification at position 1 of the quinolone moiety.
4. Fragmentation and/or modification of the dialkylamino chain/piperazinyl chain at C-7 of the quinolone moiety— side-chain degradation.
5. Decarboxylation at C-2 position on the quinolone ring.

The order in which the fragmentation mode is facilitated depends on the type of FQ. For lomefloxacin, the evidence published in the literature points to a selective reductive defluorination at position 8 as the main mode in which the major photofragments are produced upon UVA irradiation, while the minor photofragments are simultaneously produced through the modification of the *N*-alkyl chain, either at the piperazinyl group at position 7, or the 1-ethyl group on the quinolone moiety (18). The subsequent generation of damaging oxygenated products (superoxide anion, hydroxyl radical, hydrogen peroxide, *etc.*), and singlet oxygen, are some of the main factors that induce the photosensitization of FQ phototoxicity.

In the case of clinafloxacin, photodechlorination at C-8 results in the formation of a very reactive carbene, which is transformed into a reactive quinine-imine and hydrogen peroxide in the presence of water and oxygen. Fenton chemistry transforms the hydrogen peroxide into damaging hydroxyl radicals. The presence of the reactive carbenes, quinine-imines and hydroxyl radicals is responsible for the observed phototoxicity and photogenotoxicity of this drug (19).

The fluorescence of lomefloxacin (with an F-atom at the eighth position) was high at the beginning of the confocal microscopy time course. The quantum yield for defluorination of lomefloxacin is quite high (0.5–0.99 in water) (11,20). The high fluorescence level at the beginning of the confocal microscopy scan in cells is consistent with a fast defluorination at position 8 that had already been completed by the beginning of the scan, as on the average 2 s elapsed before the start of the scan. The gradual decrease during the confocal microscopy scan may represent the much slower defluorination at the sixth position, similar to the only dehalogenation process available to moxifloxacin.

The initial photochemical step in the UVA photofragmentation of BAYy3118, involves the abstraction of a chloride radical from the C-8 position leading to the formation of a highly reactive radical intermediate (possibly a reactive carbene) in addition to other photodegradation products (21). The mechanism proposed for the phototoxicity induced by this drug involves the generation of toxic oxygen radicals which attack biological systems, such as mitochondria, and the degree of cell damage depends on a balance between the amount of toxic oxygen radicals generated from the (photodechlorinated) quinolone ring, plus the flux of the UVA photons, and the scavenging activity of vitamin E, and possibly other biological defense mechanisms. Singlet oxygen does not seem to be involved in the phototoxic behavior induced by either BAYy3118 or lomefloxacin (22).

Based on this study and the literature, moxifloxacin is very photostable, and does not induce as much phototoxicity as the other compounds studied here (21,23,24). The seventh position of the fluoroquinolone ring is substituted by an octahydropyrrolo[3,4-*b*] pyridine moiety, and its C-8 position is modified with a methoxy group (–OCH₃), which significantly reduces its ability to induce phototoxicity. The phototoxic species that this drug produces are derived from the production of the cation radical MOX^{•+} when the drug is photoionized during UVA irradiation. The cation radical MOX^{•+} with a lifetime of 80 ns (21) does not present high phototoxicity, presumably because this cation radical, and all the charged species produced in the photoionization process are scarcely reactive with biological targets due to their fast

recombination, or because their intrinsic reactivity is low. The quantum yield of photoionization, reported as 0.4 (21), is quite high. Furthermore, the phototoxicity attributable to the singlet oxygen that might be produced by the very short-lived triplet state of moxifloxacin would be very low indeed.

The lysosome environment is more acidic (pH *ca* 4–5) than other cell regions (17). In addition, lysosomes were reported to accumulate zwitterionic quinolones that could not leave due to the lysosomal inner membrane charge (25). Lysosomes showed the brightest fluorescence in confocal experiments, and the association of the brightest fluorescence with lysosomes was confirmed by using another specific fluorescent marker, LysoTracker (Fig. 4). These results suggest that lysosomes may be important targets for phototoxicity exerted by halogenoquinolones, consistent with previous studies carried out using a microspectrofluorometer centered around an inverted microscope: in 1999 Ouedraogo *et al.* reported the decay of the FLQ fluorescence and its localization in lysosomes in HS68 human fibroblasts (26). In addition, there may have been a much smaller uptake into the nucleus, as DNA damage has been seen previously (9,11,22,27,28). Recently selected halogenated quinolones have also been observed to preferentially accumulate in the lysosomes and the mitochondria in human lens epithelial cells (unpublished data).

Using laser scanning confocal microscopy, in this study, we determined the temporal and spatial photoactivation of FLQs in real time in live cells, in addition to the bleaching of the fluorescence. In addition, we used a much lower concentration of 20 μ M, due to the increased sensitivity of confocal microscopy, as compared with 100 or 250 μ M FLQ, a 5- to 10-fold improvement over the previous studies by Ouedraogo *et al.* (26). To improve our understanding of the phototoxic and photocarcinogenic effect, we used human epidermal HaCaT keratinocytes, the first layers of cells targeted by UV irradiation in the presence of FLQs.

The laser scanning confocal microscopy results for these quinolones in cells demonstrate, first, that they are indeed taken up by HaCaT cells, and second, that they are localized mainly in the lysosomes. The temporal profiles are consistent with dehalogenation of cinafloxacin, lomefloxacin and Bay3118. Their temporal fluorescence *in vitro* further clarifies the difference in phototoxicity noted for these compounds: Moxifloxacin has been found to be much less phototoxic than Bay 3118, lomefloxacin and cinafloxacin (21,23,24). Their fluorescence provides a convenient way to monitor their presence within cells and to follow their photoreactivity during irradiation.

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Figure 1.
Chemical structures of fluoroquinolones.

Figure 2.
UV-Vis absorption spectra of fluoroquinolones (15 μ M, $d = 1.0$ cm).

Figure 3.

Fluorescence emission spectra ($\lambda_{\text{ex}} = 350 \text{ nm}$) of fluoroquinolones ($15 \mu\text{M}$) in the aqueous solutions (pH 7.4).

Figure 4.

Subcellular localization of FLQs in lysosomes. HaCaT cells were incubated with the indicated FLQs and LysoTracker for 1 h. Visualization of intracellular fluorescence of HaCaT cells using filter sets specific for FLQs (in blue; Ex/Em, 364 nm/385–545 nm), the LysoTracker (in red; Ex/Em, 543 nm/BP 560–615 nm) and the corresponding superimposed image using confocal microscopy. Purple showed the localization of FLQs in lysosomes.

Figure 5.

Effect of pH (4.3 and 7.4) on the fluorescence emission spectra ($\lambda_{\text{ex}} = 350 \text{ nm}$) of fluoroquinolones ($15 \mu\text{M}$) in the aqueous solutions.

Figure 6. Confocal microscopy measurement of quinolone fluorescence in human keratinocyte (HaCaT) cells under UV irradiation as a function of time. HaCaT cells were incubated with FLQs as in Fig. 4 and fluorescence was monitored using confocal microscopy.