Ciprofloxacin–Photoswitch Conjugates: A Facile Strategy for Photopharmacology

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Supporting Information

ABSTRACT: Photopharmacology aims to locally treat diseases and study biological processes with photoresponsive drugs. Herein, easy access to photoswitchable drugs is crucial, which is supported by simple and robust drug modifications. We investigated the possibility of creating drugs that can undergo remote activation and deactivation with light, by conjugating molecular photoswitches to the exterior of an existing drug in a single chemical step. This facile strategy allows the convenient introduction of various photochromic systems into a drug molecule, rendering it photoresponsive. To demonstrate the feasibility of this approach, two photoswitch-modified ciprofloxacin antibiotics were synthesized. Remarkably, for one of them a 50-fold increase in activity compared to the original ciprofloxacin was observed. Their antimicrobial activity could be spatiotemporally controlled with light, which was exemplified by bacterial patterning studies.

INTRODUCTION

The emerging field of photopharmacology focuses on the development of photoresponsive drugs.1 The activity of these drugs can be externally controlled with light, by switching between two or more isomeric states.1 Photoresponsive drugs offer the prospect for clinical applications, where a drug might be locally activated inside the body to circumvent systemic side effects as exemplified by photoswitchable sulfonyleurea derivatives used to externally control insulin release2 and by photoswitchable antibiotics3 and cytotoxic agents applied to control cellular growth.4,5 Other possible clinical applications include vision restoration6,7 and pain-perception regulation.8–10 Furthermore, the possibility to alter a drug’s activity with light can be useful as a research tool to study biological processes as shown for enzyme activity,11–14 GPCR modulation,15 neural functioning,16,17 and channel protein characterization.18,19 The in vivo application of photopharmacological agents could be enabled with recently reported visible-light switchable photochromic systems.20–24

The most commonly employed method in photopharmacology to design a photoswitchable bioactive compound is the incorporation of a photoswitch into the pharmacophore of a drug molecule or into the spacer linking two pharmacophores.1,25 The photoswitches that are predominantly used for this purpose are azobenzenes2,3,9,10,26,27 and diarylethenes11–13 because their aromatic molecular structure lends itself perfectly to incorporation into most pharmacophores and pharmacophore spacers. One particular approach aims at substituting stilbenes, diaryl amides, and diaryl ethers in a drug’s pharmacophore with azobenzenes. This has been defined as “azologization” by Trauner and co-workers28 and was applied successfully.2,28

However, incorporation of a photoswitch into a drug’s pharmacophore often requires many challenging synthetic steps and the number of photoswitches that are applicable for this purpose is limited. For example, the molecular structure of switches like spiropyrans is bulky19,29 and therefore much harder to incorporate in the design of an existing pharmacophore. Nonetheless, spiropyrans exhibit a highly pronounced change in polarity upon photoisomerization, switching from a bulky, noncharged spiropyran to a planar zwitterionic merocyanine state.29,30 When incorporated in a drug, such a change is anticipated to result in a pronounced difference in biological activity between the two isomers, rendering this photoswitch a suitable candidate for application in photopharmacology.

Therefore, we here investigated the possibility of obtaining photopharmacological agents by conjugating the photoswitch
to the exterior of the pharmacophore. We show that it is possible to synthetically modify an existing drug in a single chemical step to obtain a photoswitchable drug. This strategy is exploited to conjugate both a spiropyran and an azobenzene to the frequently prescribed broad-spectrum antibiotic ciprofloxacin. The resulting distinct isomers of these photoswitchable antibiotics were compared in their photochemistry and biological activity. It was found that the spiropyran-bearing

Figure 1. Molecular structure of ciprofloxacin (CP) and its photoswitchable analogues. (A) A carboxylic acid-bearing photoswitch is transformed into the corresponding acyl chloride and conjugated to ciprofloxacin. (i) TEA, DCM, 0 °C to rt, 16 h. (B) Structure of spirofloxacin that can be switched from its spiropyran form to its merocyanin form upon λ = 365 nm light irradiation and can be switched back upon visible-light irradiation or thermal relaxation. (C) Structure of azofloxacin that undergoes trans–cis isomerization upon λ = 365 nm light irradiation and cis–trans isomerization upon visible-light irradiation or thermal relaxation.

Figure 2. Photochemical behavior of spirofloxacin and azofloxacin. (A) UV–vis absorption spectra of spirofloxacin. (B) Thermal isomerization of the merocyanin state to the thermodynamically stable spiropyran form of spirofloxacin. The absorbance was measured at λ = 555 nm. (C) Photoswitching cycles of spirofloxacin by alternating between λ = 365 nm (blue bars) and 530 nm (green bars) irradiation, observed by monitoring the absorbance at λ = 555 nm. (D) UV–vis absorption spectra of azofloxacin. (E) Thermal cis–trans isomerization of azofloxacin. The absorbance was measured at λ = 326 nm. (F) Photoswitching cycles of azofloxacin by alternating between λ = 530 nm (green bars) and 400 nm (blue bars) irradiation, observed by monitoring the absorbance at λ = 326 nm. Spirofloxacin and azofloxacin were examined at a concentration of 20 μM in water.
antibiotic exhibited a substantial difference in activity between both photoisomers when applied to Gram-negative *Escherichia coli*. Interestingly, the azobenzene-bearing antibiotic had a significant difference in antimicrobial activity between its two photoisomeric forms when applied to the Gram-positive *Micrococcus luteus*, and was found to be ∼50 times more active than the unmodified antibiotic. This underlines the importance of exploring different photoswitches and decide which one fits best for the intended purpose, which is enabled by the presented strategy: installing photoswitches into drugs in a single synthetic step. Furthermore, bacterial patterning experiments are presented to demonstrate the spatiotemporal resolution obtained with the photoswitch–drug conjugates and emphasize the potential for localized activation with the presented approach.

## RESULTS AND DISCUSSION

Drugs bear various functional groups in their molecular structure. While these groups are often important for maintaining biological activity, by careful examination of SARs it becomes clear to what extent they can be modified. Functional groups offer exquisite handles for ligating small molecules as shown for chemical tags in the case of activity based protein profiling (ABPP). We envisioned that they could also be used for conjugating photoswitches to the drug molecule.

To study the feasibility of this approach, we choose ciprofloxacin as the target drug molecule. This synthetic, broad-spectrum antibiotic is a frequently prescribed drug and its molecular structure bears several functional groups. SAR studies showed that the secondary amine in the piperazine ring of ciprofloxacin (Figure 1A) can be modified without a major loss of antimicrobial activity: this position was therefore chosen for conjugation with photoswitches.

Two photoswitches were chosen for this purpose: a spiropyran and an azobenzene. We hypothesized that conjugation of the spiropyran to the exterior of ciprofloxacin might result in a photosensitive drug with a large difference in activity between its two photoisomeric forms, because of its pronounced change in molecular properties upon photoswitching. Azobenzene has proven to be a privileged photo-switch for the use in photopharmacology, and conjugation of the switch to the exterior of ciprofloxacin might result in an antibiotic with photoswitchable activity.

Carboxylic acid-modified spiropyran and azobenzene were used for ligation. The acid functionalities were readily transformed into the corresponding acyl chloride and sequentially conjugated to the secondary amine of ciprofloxacin (Figure 1A). The resulting photoresponsive antibiotics were named spirofloxacin (Figure 1B) and azofloxacin (Figure 1C).

Next, the photochemical behavior of the two compounds was studied using UV–vis spectroscopy and RP-HPLC. Colorless spirofloxacin shows a strong absorbance in the UV region of the UV–vis spectrum (Figure 2A). Exposure to λ = 365 nm light results in the appearance of an absorption band around λ = 550 nm, which is characteristic for the formation of the colored merocyanine state (Figure 2A). The spiropyran state is the thermodynamically stable form, and by monitoring the spectral evolution at λ = 555 nm, the half-life of the merocyanine state was determined and was found to be ∼17 h (Figure 2B).

Reversible photochromism of spirofloxacin in water was tested, by alternating between λ = 365 and 530 nm irradiation. After each round of irradiation, significant fatigue was observed (Figure 2C). This may be attributed to the instability of the merocyanine structure in aqueous environment under visible-light irradiation, since it undergoes a retro-aldol reaction to form a Fischer’s base and 4-nitro-salicylaldehyde, as reported by Hilvert and co-workers. This instability limits the use of spirofloxacin to a single round of switching. However, for its employment as a potential photopharmacological agent, a single round of activation is often sufficient.

Azofloxacin has an absorption maximum around λ = 325 nm (Figure 2D), which is characteristic for trans-azobenzene. When the azofloxacin solution was irradiated with λ = 365 nm light, the absorption at λ = 325 nm decreased and simultaneously an absorption band appeared around λ = 430 nm, which is characteristic for cis-azobenzene. Overtime, cis-azobenzene thermally reverts back to trans-azobenzene. The half-life of the cis-form was determined by monitoring the absorption of azofloxacin at λ = 326 nm and was found to be ∼4 h (Figure 2E). Reversible switching between trans- and cis-azofloxacin in water could be performed >10 times by alternating between λ = 400 and 530 nm irradiation, without any observable fatigue (Figure 2F).

Both spirofloxacin and azofloxacin consist of a mixture of two photoisomers. The ratio between these isomers, which can be altered by exposure to light, was determined using RP-HPLC. These experiments revealed that spirofloxacin consisted of 74% of the spiropyran isomer before exposure to λ = 365 nm light. After λ = 365 nm light irradiation, 82% of the merocyanine was present (Table 1 and Figure S1). This preferential formation of the zwitterionic isomer of spirofloxacin after irradiation and the accompanied large changes in properties were anticipated to significantly alter the observed antimicrobial activity of the compound.

Azofloxacin exists in trans and cis states. Before exposure to λ = 365 nm light, the sample consisted of 100% trans isomer. After irradiation, 61% cis isomer was present in the sample (Table 1 and Figure S2). A possible explanation for the relatively low amount of cis isomer after irradiation might be aggregation of the azobenzene containing molecules in aqueous environment as we recently observed for other functionalized azobenzenes.

The antimicrobial activity of spirofloxacin and azofloxacin was determined by performing minimum inhibitory concentration (MIC) tests on *E. coli* CS1562 and *M. luteus* ATCC.
 Spiroloxacin was shown to have a MIC of 1.25 μM on *E. coli*, before exposure to light, when it was mostly in the spiropyran state. Remarkably, when spiroloxacin was irradiated with $\lambda = 365$ nm light, prior to incubation with the bacteria, a MIC of 0.625 μM was found (Table 1, Figure 3A, and Figure S3). This implies that spiroloxacin has higher antibacterial activity when it is in its light-induced zwitterionic merocyanine state. The difference in activity of spiroloxacin might be caused by its pronounced change in dipole moment from 2 to 5 D (spiropyran) to 20 D (merocyanine), which is likely to affect cellular uptake and drug–receptor interactions. No significant difference in antibacterial activity was observed when spiroloxacin was tested on Gram positive *M. luteus* (Table 1 and Figure S4).

Azofloxacin showed to have a MIC of 0.250 μM before and after irradiation with $\lambda = 365$ nm light (Table 1 and Figure S5) when tested on *E. coli*. However, when azofloxacin was tested on *M. luteus*, a clear difference in antimicrobial activity was observed before and after irradiation (Table 1, Figure 3B, and Figure S6). The thermally adapted form had a MIC value of 0.250 μM, whereas the light-exposed form had a MIC of 0.500 μM. This indicates that the trans isomer has higher antibacterial activity than the cis isomer and this activity can be dynamically changed by exposing the compound to light. Remarkably, the activity of azofloxacin on *M. luteus* is almost 50 times higher than that of native ciprofloxacin. This result, together with earlier reports on photocontrolled mast cell-stabilizing agents, challenges the notion that ligation of a photoswitch to a drug inherently decreases its activity. In this case, the increased activity might be due to enhanced cellular uptake caused by the addition of the hydrophobic azobenzene moiety.

As a control experiment, the MIC value of the native drug ciprofloxacin was also determined, and no change in activity was found before and after irradiation with $\lambda = 365$ nm (Figure S7). This indicates that the observed change in activity of spiroloxacin and azofloxacin before and after irradiation indeed stems from the photoisomerization process. Furthermore, control experiments showed that nonligated spiropyran and azobenzene exhibited no antibacterial activity (Figures S8 and S9).

Next, bacterial patterning experiments were performed to showcase that significant spatiotemporal resolution can be obtained with photoswitch–drug conjugates. An agar plate was prepared containing spiroloxacin (300 nM). A mask was placed on top of the plate (Figure 4A) and the plate was illuminated with $\lambda = 365$ nm light for 30 min. The mask was removed and the plate was inoculated with *E. coli* and incubated overnight at 37 °C. Bacterial growth was only observed at the area of the plate that was covered by the mask (Figure 4B). At the light-exposed area, the activity of spiroloxacin was switched on and bacterial growth was inhibited. This experiment underlines the potential of using light-responsive drugs for localized activation. Furthermore, it demonstrates that the acquired difference in activity between the two photoisomers is large enough to optically control bacterial growth in time and space.

## CONCLUSIONS

We have shown the possibility of modifying an existing drug in a single step to render it photoresponsive. Conjugation of two different photoswitches was accomplished and the photochemistry and biological activity of the two photoswitchable drugs were compared. We found a significant difference in bioactivity between the two photoisomers of the spiropyran-modified antibiotic when tested on *E. coli*. Interestingly, a difference in activity between the two isomers of the azobenzene-modified antibiotic was found when tested on Gram positive *M. luteus*, but not on Gram negative *E. coli*. Notably, the overall activity of azofloxacin on *M. luteus* was enhanced by exposure to light. This indicates that the observed change in activity of spiroloxacin and azofloxacin before and after irradiation indeed stems from the photoisomerization process. Furthermore, control experiments showed that nonligated spiropyran and azobenzene exhibited no antibacterial activity (Figures S8 and S9).

### Figure 3. Growth rates of *E. coli* CS1562 at increasing concentrations of spiroloxacin (A) and of *M. luteus* at increasing concentrations of azofloxacin (B) in their dark-adapted form (blue) and $\lambda = 365$ nm light iradiated form (red). Error bars show s.d. calculated from measurements in triplicate.

### Figure 4. Spatiotemporal patterning of *E. coli* CS1562 with spiroloxacin (300 nM). (A) Mask used to cover part of the agar plate during illumination with $\lambda = 365$ nm light. (B) Result of the patterning experiment after incubation for 16 h at 37 °C. Bacterial colonies are present only in the area that was not illuminated. N.B. inoculation occurred after $\lambda = 365$ nm light exposure.
increased almost 50-fold as compared to native ciprofloxacin. Bacterial patterning studies were performed with spirofl oxacin to illustrate the spatiotemporal resolution of the obtained photoswitchable antibiotics, underlining the potential for localized activation of drugs.

The presented approach for conjugating photoswitches to the exterior of an existing drug in a single step allows for easy access to photoswitchable drugs, avoiding laborious synthetic steps, and offering ample opportunities to explore new targets in photopharmacology.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00591.

Experimental procedures, patterning experiments, photochemical data (UV–vis spectra and HPLC data), MIC tests, synthetic schemes and procedures, and NMR and HRMS spectra of all final products (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge generous support from The Netherlands Organization for Scientific Research (NWO–CW, Top grant to B.L.F. and VIDI grant no. 723.014.001 for W.S.), the Ministry of Education, Culture and Science (KNAW), the Royal Netherlands Academy of Arts and Sciences Science (Gravitation program 024.001.035) and the European Research Council (Advanced Investigator Grant, no. 227897 to B.L.F.). We also thank T. Tiemersma-Wegman for ESI-MS analyses.

REFERENCES


(27) Bandara, H. M. D., and Burdette, S. C. (2012) Photo- 

isomerization in different classes of azobenzene. Chem. Soc. Rev. 41, 

1809–1825.

(28) Schoenberger, M., Damijonaitis, A., Zhang, Z., Nagel, D., and 

Trauner, D. (2014) Development of a new photochromic ion channel 


518.

(29) Brieke, C., Rohrbach, F., Gottschalk, A., Mayer, G., and Heckel, 


8476.


(32) Heal, W. P., Dang, T. H. T., and Tate, E. W. (2011) Activity- 


(33) Hooper, D. C., and Wolfson, J. S. (1993) Quinolone 

antimicrobial agents, 2nd ed., American Society for Microbiology, 

Washington, DC.

(34) Stafforst, T., and Hilvert, D. (2009) Kinetic characterization of 


(35) Veleva, W. A., van der Toorn, M., Szymanski, W., and Feringa, 

B. L. (2013) Design, synthesis, and inhibitory activity of potent, 

photoswitchable mast cell activation inhibitors. J. Med. Chem. 56, 

4456–4464.

(36) Veleva, W. A., Stuart, M. C. A., Szymanski, W., and Feringa, B. 

L. (2013) Light-triggered self-assembly of a dichromonyl compound in 


(37) Wiegand, I., Hilpert, K., and Hancock, R. E. W. (2008) Agar and 

broth dilution methods to determine the minimal inhibitory 

concentration (MIC) of antimicrobial substances. Nat. Protoc. 3, 

163–175.

(38) Austin, E. A., Graves, J. F., Hite, L. A., Parker, C. T., and 


biosynthesis by Escherichia coli K-12: insertion mutagenesis of the rfa 


(39) Hirai, K., Aoyama, H., Irikura, T., Iyobe, S., and Mitsuhashi, S. 

(1986) Differences in susceptibility to quinolones of outer membrane 

mutants of Salmonella typhimurium and Escherichia coli. Antimicrob. 


(40) Delcour, A. H. (2009) Outer membrane permeability and 

antibiotic resistance. Biochim. Biophys. Acta, Proteins Proteomics 1794, 

808–816.