

Affinity of Fluoroquinolone-Safirinium Dye Hybrids to Phospholipids Estimated by IAM-HPLC

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Abstract:

Nowadays, fluoroquinolones (FQs) constitute one of the most important classes of antibiotics. FQs are used to treat infections caused by Gram-positive and Gram-negative species. A set of fluoroquinolone-Safirinium dye hybrids has been synthesized in our laboratory as potential new dual-action antibacterial agents. In the present study we have evaluated how such a modification influences the affinity of FQs to phospholipids. The immobilized artificial membrane (IAM) high-performance liquid chromatography (IAM-HPLC) was used as a tool for the determination of phospholipids partitioning. The obtained results indicate that the fluoroquinolone-Safirinium dye hybrids, especially the SafiriniumP conjugates, display significantly lower affinity to phospholipids than the parent FQs. Despite the fact that the hybrid structures comprise a quaternary nitrogen atom and hence are permanently charged, the attractive electrostatic interactions between the solutes and negatively charged phospholipids do not occur or occur at a lesser extent than in the case of the unmodified FQs. Since affinity of FQs to phospholipids involves molecular mechanism, which is not entirely determined by lipophilicity, assessment of phospholipid partitioning should be considered at the early stage of the development of new FQ antibiotics.

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Communication

Affinity of Fluoroquinolone–*Safirinium* Dye Hybrids to Phospholipids Estimated by IAM-HPLC

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Abstract: Nowadays, fluoroquinolones (FQs) constitute one of the most important classes of antibiotics. FQs are used to treat infections caused by Gram-positive and Gram-negative species. A set of fluoroquinolone–*Safirinium* dye hybrids has been synthesized in our laboratory as potential new dual-action antibacterial agents. In the present study we have evaluated how such a modification influences the affinity of FQs to phospholipids. The immobilized artificial membrane (IAM) high-performance liquid chromatography (IAM-HPLC) was used as a tool for the determination of phospholipids partitioning. The obtained results indicate that the fluoroquinolone–*Safirinium* dye hybrids, especially the *Safirinium P* conjugates, display significantly lower affinity to phospholipids than the parent FQs. Despite the fact that the hybrid structures comprise a quaternary nitrogen atom and hence are permanently charged, the attractive electrostatic interactions between the solutes and negatively charged phospholipids do not occur or occur at a lesser extent than in the case of the unmodified FQs. Since affinity of FQs to phospholipids involves molecular mechanism, which is not entirely determined by lipophilicity, assessment of phospholipid partitioning should be considered at the early stage of the development of new FQ antibiotics.

Keywords: fluoroquinolones; fluoroquinolone–*Safirinium* dye hybrids; immobilized artificial membrane

1. Introduction

Fluoroquinolones (FQs) are one of the most commonly prescribed classes of antibiotics and show a broad spectrum of antibacterial activity, including against both Gram-positive and Gram-negative bacterial species. FQs originate from the nalidixic acid structure, and their major modifications include the introduction of a fluorine atom at the position 6 and the addition of a piperazine moiety at the position 7 of the quinolone ring [1]. A number of research groups develop hybrid compounds based on FQs structures linked to other antibacterial agents [2]. This concept of “dual-action drugs” has been gaining popularity since such hybrid drugs show synergistic molecular action, which enhances affinity and efficacy when compared to parent drugs. In addition, the dual-drug conjugates demonstrate the capability to reduce cross-resistance, and one part of a hybrid may counterbalance the known side effects associated with the other part of the drug [2,3].

Our research group has reported the design and synthesis of fluorescent 1,2,4-triazolo[4,3-*a*]pyridin-2-ium and 1,2,4-triazolo[4,3-*a*]quinolin-2-ium carboxylates that were named *Safirinium P* (pyridine

derivatives, *SafP*) and *Safirinium Q* (quinolone derivatives; *SafQ*) dyes, respectively (Figure 1) [4,5]. Their unique features, i.e., lack of toxicity, extraordinary water solubility, adjustable lipophilicity, and relatively low production cost, prompted us to utilize this class of compounds as amino acids and peptide-labeling reagents [6]. The novel *Safirinium* (*Saf*) dyes were also applied as human skin structures and bacterial spore-staining agents [4]. Moreover, due to the presence of permanent positive charge within the triazolium moiety, *Saf* dyes were employed as ionization tags suitable for peptide identification [6].

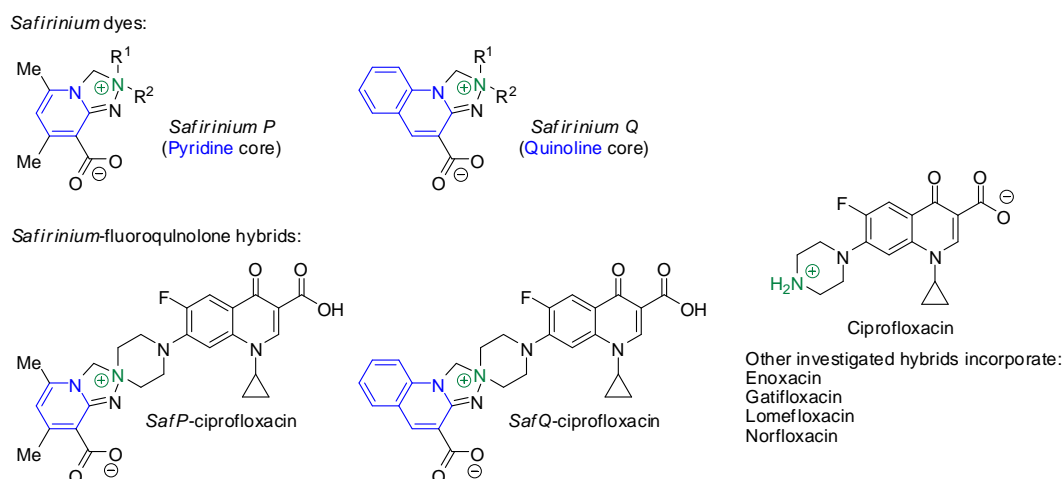


Figure 1. General structures of *Safirinium* dyes and their hybrids exemplified by ciprofloxacin, *SafP*-ciprofloxacin and *SafQ*-ciprofloxacin derivatives.

Recently, a set of fluoroquinolone–*Safirinium* dye hybrids has been synthesized in our laboratory as new dual-acting hydrophilic antibacterial agents [7]. The concept assumed that conjugation of *Saf* dyes to antibiotics would result in the development of new antibacterials that would exhibit dual mode of action: perturbation of bacterial lipid membranes caused by the presence of the quaternary nitrogen atom and inhibition of bacterial type II topoisomerases elicited by the fluoroquinolone portion. The synthesized structures demonstrate more hydrophilic characteristics than the parent FQs, which was shown in our previous investigations based on reversed phase liquid chromatography (RPLC) and micellar electrokinetic chromatography (MEKC) [8]. The importance of drug candidate characterization in terms of their physicochemical properties is critical, since these analyses support selection of the most promising compounds [9–12]. Although, lipophilicity is commonly applied for prediction of absorption, distribution, metabolism, and excretion (ADME) properties, usually with the octanol–water system or RPLC, several studies pointed out that these systems are oversimplified to simulate membrane partitioning [13]. Generally, two main differences between membranes and *n*-octanol are quoted. The first discrepancy refers to anisotropy, since membranes are anisotropic phases where phospholipids are spatially ordered, whereas *n*-octanol is an isotropic phase [14]. The second difference pertains to electric character of molecules, since membrane phospholipids are electrically charged, while *n*-octanol is neutral [14]. For this reason, partitioning systems containing phospholipids as the organic phase are more suitable to mimic the partitioning of ionized drugs into biological membranes. Although affinity to phospholipids can be determined using liposomes or cell culture techniques, nowadays, the most popular approach involves immobilized artificial membrane HPLC (IAM-HPLC). Advantages of IAM-HPLC include ease of automation, quickness, and sufficient lab-to-lab reproducibility [15–20].

This study is a continuation of our research program focused on the assessment of physicochemical properties of fluoroquinolone–*Safirinium* dye hybrids that condition their biological activity [7,8]. The main goal was to evaluate the affinity of fluoroquinolone–*Safirinium* dye hybrids to phospholipids using IAM-HPLC, and to compare the obtained results with those assessed for the parent FQs. Based on

theoretical assumption, two research hypotheses were verified during the study. The first assumed that the fluoroquinolone–*Safirinium* dye hybrids interact stronger with phospholipids than unmodified FQs since the hybrids are permanently positively charged. The alternative hypothesis presupposed that fluoroquinolone–*Safirinium* dye hybrids display lower affinity to phospholipids when compared to parent FQs due to their significantly lower lipophilic properties.

2. Results and Discussion

IAM-HPLC was introduced by Pidgeon in 1989 [21], and it was proposed as an alternative for liposome's partitioning. Since that time, the interest in IAM-HPLC increased regularly, as evident from a large number of publications. Nevertheless, there is no one widely recognized protocol for IAM retention measurements in the medical chemistry community [13]. In our study, we have chosen the chromatographic hydrophobicity indices (CHI_{IAM}) approach proposed by Valko and co-workers [22], which involves gradient elution using an IAM column. This method is routinely used for the assessment of drug candidates by the GlaxoWellcome Medicines Research Centre [23]. The determined CHI_{IAM} indexes correspond to the fraction of acetonitrile, which leads to equal analyte concentrations in both stationary and mobile phases [24].

The retention data of the investigated fluoroquinolone–*Safirinium* dye hybrids and the parent FQs are given in Table S2, whereas the determined CHI_{IAM} indexes are presented in Table 1.

Table 1. The experimental chromatographic hydrophobicity indices (CHI_{IAM}) and $CHI_{pH7.4}$ chromatographic hydrophobicity indices together with minimum inhibitory and bactericidal concentrations (MIC/MBC) (g/mL) * determined for the analyzed compounds.

Compound	CHI_{IAM}	$CHI_{pH7.4}$	MIC/MBC <i>S. aureus</i> ¹	MIC/MBC <i>S. epidermidis</i> ²	MIC/MBC <i>P. aeruginosa</i> ³	MIC/MBC <i>P. vulgaris</i> ⁴
Ciprofloxacin	19.70	35.61	0.25/0.5	0.25/0.25	0.5/0.5	0.008/0.008
<i>SafP</i> –ciprofloxacin	2.08	25.13	8/16	4/16	32/>512	1/2
<i>SafQ</i> –ciprofloxacin	7.55	33.93	8/8	8/8	64/64	2/2
Enoxacin	21.06	34.43	1/1	0.5/2	0.5/0.5	0.032/0.063
<i>SafP</i> –enoxacin	0.62	18.13	64/128	64/128	256/>512	0.25/0.5
<i>SafQ</i> –enoxacin	3.90	30.61	64/64	32/32	64/64	2/2
Gatifloxacin	19.69	40.42	0.25/0.25	0.125/0.25	0.25/0.25	0.008/0.008
<i>SafP</i> –gatifloxacin	3.50	30.43	4/8	4/4	64/128	0.5/0.5
<i>SafQ</i> –gatifloxacin	9.22	39.50	8/8	2/8	128/128	0.5/2
Lomefloxacin	16.52	34.81	1/1	1/2	1/1	0.008/0.008
<i>SafP</i> –lomefloxacin	n.d.	24.49	128/128	64/64	128/128	2/2
<i>SafQ</i> –lomefloxacin	4.41	34.71	4/4	2/2	2/2	0.063/0.063
Norfloxacin	19.18	35.08	0.25/0.25	0.5/2	0.25/0.25	0.016/0.016
<i>SafP</i> –norfloxacin	1.94	24.63	64/64	32/64	128/>512	1/1
<i>SafQ</i> –norfloxacin	7.96	34.56	2/2	4/4	1/1	0.125/0.125

*—the data assessed in our previously study [7]; n.d.—not detected; CHI_{IAM} —chromatographic hydrophobicity indices determined with IAM-HPLC; $CHI_{pH7.4}$ —chromatographic hydrophobicity indices determined with reversed phase HPLC at physiological pH conditions; ¹—*Staphylococcus aureus*; ²—*Staphylococcus epidermidis*; ³—*Pseudomonas aeruginosa*; ⁴—*Proteus vulgaris*.

During this study, five FQs (ciprofloxacin, enoxacin, gatifloxacin, lomefloxacin, norfloxacin) and their *Safirinium*–*P* (pyridine core) and *Safirinium*–*Q* (quinoline core) derivatives have been examined. The chemical names and structures of the studied quinolone derivatives are presented in Table S1. The obtained CHI_{IAM} indexes of FQs antibiotics are quite comparable and range from 16.52 (lomefloxacin) to 21.06 (enoxacin). Analogous results have been reported by Barbato and co-workers, who described very similar retention behavior of zwitterionic ampholytes FQs [25] in analyses run using the IAM.PC.MG column. Moreover, Barbato and co-workers postulated that IAM retention is not conditioned by lipophilicity, but that it is governed by other recognition forces, probably of electrostatic nature [25–28]. In order to evaluate this hypothesis, we determined CHI parameters under physiological pH conditions using the typical RP-HPLC approach. In general, the lack of correlation between the lipophilicity indexes $CHI_{pH7.4}$ and CHI_{IAM} should be emphasized. However, upon exclusion of unmodified FQs from the dataset, a linear relationship between $CHI_{pH7.4}$

and CHI_{IAM} parameters is evidenced for *Safirinium* derivatives ($r = 0.908$). These results are presented graphically in Figure 2.

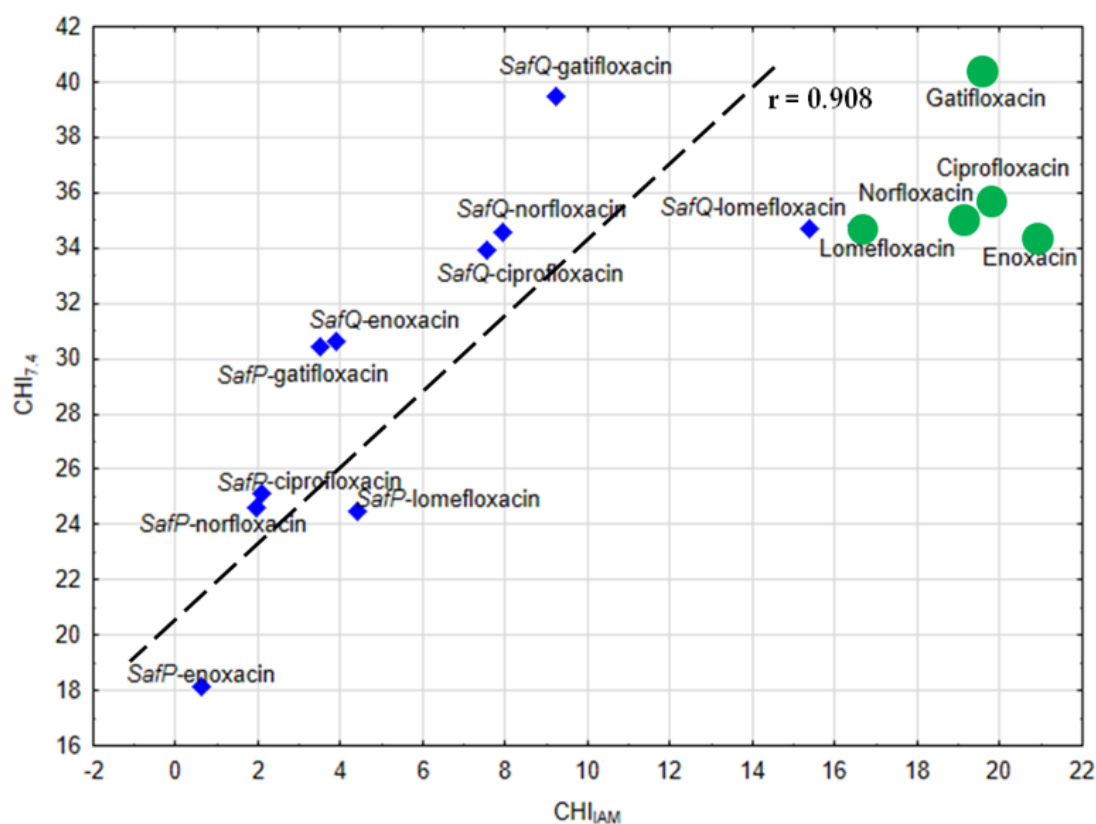


Figure 2. Scatterplots comparing $CHI_{pH7.4}$ and CHI_{IAM} parameters determined for parent fluoroquinolones (FQs; marked green) and *Safirinium* derivatives (marked blue).

However, it should be noted that the CHI_{IAM} values estimated for the hybrids (from 1.94 to 9.22) are significantly lower than those established for the parent FQs (from 16.52 to 19.69). Considering the fact that the newly synthesized fluoroquinolone–*Safirinium* hybrids are permanently charged, the molecular retention mechanism on the IAM column should promote higher partitioning of such analytes into negatively charged phospholipids since the electrostatic interactions would be the main factor that determines the affinity of charged molecules to phospholipids. Unexpectedly, the obtained results contradicted this theoretical assumption. Hence, both *Safirinium*–*P* and *Safirinium*–*Q* conjugates proved noticeably lower affinities to phospholipids than the parent FQs. However, while the pyridine derivatives practically lost their affinities for the phosphatidylcholine layer (from 0.62 to 3.50), the quinolone congeners were to some extent retained on the IAM column (from 3.90 to 9.22). These results indicate that lipophilicity is the major retention factor for permanently ionized *Safirinium*–FQ conjugates. This observation can be further supported by biological activity studies and lipophilicity assessments by means of MEKC, where a significant correlation between retention in MEKC and antibacterial activity was found [3]. Hence, in most cases, less lipophilic *SafP* derivatives exhibited lower antibacterial activities than their *SafQ* counterparts and unmodified FQs. *SafP*–lomefloxacin was not detected in IAM experiments since this compound presumably migrated with the front of the mobile phase. The most striking result obtained within this study pertains to *Safirinium*–*Q* hybrids, which, despite showing only slightly lower RP-HPLC lipophilicity indexes ($CHI_{pH7.4}$) than the parent FQs (gatifloxacin 40.42 $CHI_{pH7.4}$ vs. *SafQ*–Gatifloxacin 39.50), proved noticeably weaker affinity to phospholipids in IAM-HPLC analyses (CHI_{IAM} of 19.69 vs. 9.22, respectively) (Table 1). These differences in retention of *Safirinium*–*Q* hybrids compared to parent FQs can be explained by

the fact that the positive charge on the quaternary nitrogen atom (Figure 1) is sterically hindered and not available for interaction with negatively charged phosphate groups of phospholipids (Figure 3). On the contrary, ionization of carboxyl groups in FQs contributes only to a minor extent to interactions with phospholipids. This finding is supported by Barbatto theory that ionization enhancement of the amino functionality within a FQ results in higher retention in IAM-HPLC [25]. Considering previously reported biological data [7], compounds with higher affinity to IAM and lipophilicity demonstrate higher antibacterial efficacy, particularly in the case of Gram-positive species, such as *Staphylococcus aureus* and *Staphylococcus epidermidis* (Table 1). It is worth emphasizing, however, that FQs can also penetrate into bacterial cells in hydrophilic manner through porin channels, which is especially true for Gram-negative bacteria [29,30]. The latter can explain noticeable activity of the tested *SafP* hybrids against *Proteus vulgaris* (Table 1).

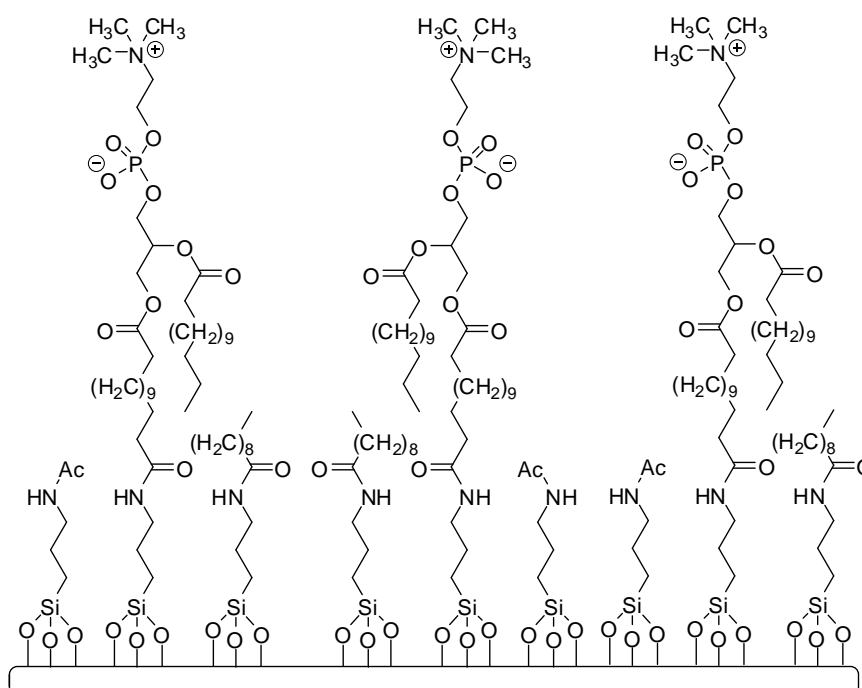


Figure 3. Amphoteric phospholipids covalently bonded to aminopropyl silica in the IAM.PC.DD2 column.

3. Materials and Methods

3.1. Reagents

Dimethyl sulfoxide (DMSO) was acquired from POCH (Gliwice, Poland). Acetonitrile HPLC grade for liquid chromatography, sodium phosphate dibasic dehydrate and sodium phosphate monobasic monohydrate were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water, obtained with the Millipore Direct-Q 3 UVWater Purification System (Millipore Corporation, Bedford, MA, USA), was used for buffer mobile phase preparation.

3.2. Analytes

The analytical standards of octanophenone, butyrophenone acetanilide, enoxacin, and gatifloxacin were provided by Alfa Aesar (Haverhill, MA, USA); acetaminophen, theophylline, benzimidazole, acetophenone, indole, norfloxacin, lomefloxacin, and ciprofloxacin were purchased from Sigma-Aldrich (Steinheim, Germany); heptanophenone, hexanophenone, valerophenone, propiophenone, acetophenone were bought from Acros Organic (Massachusetts, United States). All the compounds listed above, with the exception of the FQs, were used as model substances in order to determine CHI indices of the studied FQs and their *SafP* and *SafQ* derivatives according to the protocol proposed

by Valko and co-workers [12]. Synthesis and purification of the hybrid quinolone-based quaternary ammonium derivatives were described previously [7]. The structures of the investigated hybrids are presented in Table S1. All studied compounds were dissolved in DMSO to obtain a concentration of 1 mg mL^{-1} , and they were stored at $2\text{--}8 \text{ }^{\circ}\text{C}$ prior to analyses.

3.3. HPLC Analysis

All HPLC experiments were carried out using a Prominence-1 LC-2030C 3D HPLC system (Shimadzu, Japan) equipped with Diode-Array Detection (DAD) and controlled by LabSolution system (version 5.90 Shimadzu, Japan). The stock solutions of solutes were diluted to obtain concentrations of $100 \text{ }\mu\text{g/mL}$, and the injected volume was $5 \text{ }\mu\text{L}$. The RP-HPLC experiments were performed on a C_{18} CORTECS column ($2.7 \text{ }\mu\text{m}$ $2.1 \times 75 \text{ mm}$; Waters; Milford, Massachusetts, USA) with a linear gradient $2\text{--}98\%$ phase B (where phase A was 10 mM phosphoric buffer at pH 7.4, and phase B was acetonitrile) at a flow rate of 0.2 mL/min . The temperature of the chromatographic column was controlled and set to $40.0 \text{ }^{\circ}\text{C}$, and analysis time was 10 min . The IAM-HPLC analyses were carried on an IAM.PC.DD2 column ($10 \times 4.6 \text{ mm}$; particle size $10.0 \text{ }\mu\text{m}$ with an IAM guard column; Regis Technologies, USA) with a linear gradient $0\text{--}85\%$ phase B (where phase A was 10 mM phosphoric buffer at pH 7.4, and phase B was acetonitrile) at a flow rate of 1.5 mL/min . The temperature of the chromatographic column was controlled and set to $30.0 \text{ }^{\circ}\text{C}$, and the analysis time was 6.5 min . The CHI indexes, both $\text{CHI}_{\text{pH}7.4}$ and CHI_{IAM} indexes of target compounds, were obtained using a calibration set of reference substances using the protocol proposed by Valko and co-workers [12]. Figure S1 presents correlations between $\text{CHI}_{\text{pH}7.4}$ and CHI_{IAM} indexes of model substances and experimentally determined retention times. The retention times and absorbance maxima of the studied FQs and fluoroquinolone–*Safirinium* dye hybrids are listed in Table S2, whereas Figure S2 presents representative chromatograms. Each HPLC analysis was run in duplicate.

4. Conclusions

The obtained results indicate that fluoroquinolone–*Safirinium* dye hybrids incorporating permanent positive charge, especially *SafP* hybrids, feature significantly lower affinities to phospholipids than the parent fluoroquinolone antibiotics. The reduction in phospholipids affinity presumably results from steric hindrance, which prevents interactions of quaternary nitrogen atoms with negatively charged phosphate groups of phospholipids. *Safirinium*–Q hybrids, despite having similar RP-HPLC lipophilicity indexes ($\text{CHI}_{\text{pH}7.4}$) to fluoroquinolone antibiotics, present significantly lower affinities to immobilized artificial membranes (CHI_{IAM}) in IAM-HPLC analyses. This observation suggests that for ionized drug candidates, the $\text{CHI}_{\text{pH}7.4}$ and CHI_{IAM} lipophilicity indexes cannot be utilized interchangeably since phospholipid partitioning involves different molecular interactions when compared to classical two-phase separation models.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2227-9717/8/9/1148/s1>, Table S1. The structures of the studied quinolone derivatives; Table S2. Retention data of fluoroquinolone–*Safirinium* dye hybrids and parent FQs obtained by RP-HPLC and IAM-HPLC. Figure S1. The plots of acknowledged chromatographic hydrophobicity indexes $\text{CHI}_{\text{pH}7.4}$ (RP) and known chromatographic hydrophobicity indexes CHI_{IAM} (IAM) vs. retention times determined experimentally for the sets of model compounds. Figure S2. IAM-HPLC chromatograms of ciprofloxacin and its *SafP* and *SafQ* derivatives.

Author Contributions: Project conceptualization, K.C. and J.F.; methodology, K.C.; software, K.C.; formal analysis, K.C.; IAM-HPLC analysis, H.K.; RP-HPLC analysis, A.A.; writing—original draft preparation, K.C. and J.F.; writing—review and editing, J.S.; visualization, K.C. and J.F.; project supervision, J.S.; project administration, K.C.; funding acquisition, W.S. All authors have read and agreed to the published version of the manuscript.

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