

Research Article

Pleiotropic Properties of Amphiphilic Dihydropyridines, Dihydropyridones, and Aminovinylcarbonyl Compounds

Martins Rucins ¹, Rufus Smits ¹, Anda Sipola ¹, Brigita Vigante ¹,
Ilona Domracheva ¹, Baiba Turovska ¹, Ruslan Muhamadejev ¹, Karlis Pajuste ¹,
Mara Plotniece ², Arkadij Sobolev ¹, Gunars Duburs ¹, and Aiva Plotniece ¹

¹Latvian Institute of Organic Synthesis, Aizkraukles 21, Riga LV-1006, Latvia

²Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Riga Stradiņš University, Dzirciema 16, Riga LV-1007, Latvia

Correspondence should be addressed to Gunars Duburs; gduburs@osi.lv and Aiva Plotniece; aiva@osi.lv

Received 23 July 2020; Revised 9 October 2020; Accepted 10 November 2020; Published 31 December 2020

Academic Editor: Sasanka Chakrabarti

Copyright © 2020 Martins Rucins et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Three groups of synthetic lipids are chosen for studies: (1) 1,4-dihydropyridines (1,4-DHPs) containing two cationic moieties and their analogues; (2) 3,4-dihydro-2(1*H*)-pyridones containing a cationic moiety; and (3) acyclic, open-chain analogues, i.e., 2-amino-3-alkoxycarbonylalkylammonium derivatives. 1,4-DHPs possessing dodecyl alkyl chains in the ester groups in positions 3 and 5 and cationic nitrogen-containing groups in positions 2 and 6 have high cytotoxicity in cancer cells HT-1080 (human lung fibrosarcoma) and MH-22A (mouse hepatoma), but low cytotoxicity in the noncancerous NIH3T3 cells (mouse embryonic fibroblast). On the contrary, similar compounds having short (methyl, ethyl, or propoxyethyl) chains in the ester groups in positions 3 and 5 lack cytotoxicity in the cancer cells HT-1080 and MH-22A even at high doses. Inclusion of fluorine atoms in the alkyl chains in positions 3 and 5 of the DHP cycle decreases the cytotoxicity of the mentioned compounds. Structurally related dihydropyridones with a polar head group are substantially more toxic to normal and cancerous cells than the DHP analogues. Open-chain analogues of DHP lipids comprise the same conjugated aminovinylcarbonyl moiety and possess anticancer activity, but they also have high basal cytotoxicity. Electrochemical oxidation data demonstrate that oxidation potentials of selected compounds are in the range of 1.6–1.7 V for cationic 1,4-DHP, 2.0–2.4 V for cationic 3,4-dihydropyridones, and 1.2–1.5 V for 2-amino-3-alkoxycarbonylalkylammonium derivatives. Furthermore, the tested cationic 1,4-DHP amphiphiles possess antiradical activity. Molecular topological polar surface area values for the tested compounds were defined in accordance with the main fragments of compound structures. The determined log*P* values were highest for dodecyl ester groups in positions 3 and 5 of the 1,4-DHP and lowest for short alkyl chain-containing amphiphiles.

1. Introduction

For a long time, liposomes have been considered to have a role in the encapsulation of toxic drugs (mainly anticancer drugs) into liposomal drug delivery systems which are supposed to be inert, having no pharmacological or chemotherapeutic activity *per se* [1]. The properties of liposomes are mainly dependent on the characteristics of their constituent lipids.

Lipids and liposomal agents as liposome-forming building blocks depending on their physicochemical properties can influence the immune system. For the development of

new liposomal systems, it is important to perform analysis on the liposome-forming lipid properties with an emphasis on toxicity to healthy tissues and immune responses [2]. Synthetic cationic amphiphilic bilayers can act as gene, vaccine, or drug carriers; at the same time, they can interact with negatively charged prokaryotic or eukaryotic cells, causing cell adhesion and loss of cell viability. Lipids and liposomes deserve to be investigated as potential drugs [3]. Cytotoxicity of cationic liposomes is a significant property to be checked [4].

Liposomes may induce oxidative damage to normal tissues [5]. Reactive oxygen species (ROS) and lipid peroxidation

products not only are cytotoxic but may also perform and modulate signal transduction in cells [6]. It was demonstrated that the levels of ROS and the activity of scavenging/antioxidant enzymes in drug-resistant cancer cells are typically increased compared to nonresistant cancer and normal cells. Thereby, multidrug-resistant (MDR) cancer cells may be more susceptible to alterations in ROS levels. Numerous studies suggest that compounds modulating cellular ROS levels can enhance MDR cancer cell death and sensitize MDR cancer cells to certain chemotherapeutic drugs [7]. It was concluded that unspecific elimination of ROS by use of low molecular mass antioxidants was not successful for disease initiation and progression. However, controlling specific ROS-mediated signaling pathways by selective targeting offers a perspective for more refined redox medicine in the future [8]. Additional antioxidant activity (antiradical activity) could be beneficial as it would protect cells and organisms in case of oxidative stress or, in general, be involved in the process of redox regulation and master switch systems [9]. In this way, 1,4-dihydropyridines, being a group of synthetic antioxidants, could be used for the modulation of cellular redox signaling. Lipids reveal cancer cell-selective cytotoxicities—they are less cytotoxic in noncancerous healthy cells [10]. Evaluation of the cytotoxicity of nanoparticles and their components is crucial for the accurate interpretation of pharmacological activities [11]. Nonviral synthetic lipid vectors may interact with biomolecules through functional moieties (biosignature), resulting in biological impacts: intrinsic genomic and nongenomic effects [12].

Our research group has developed liposome-forming cationic 1,4-dihydropyridine (1,4-DHP) amphiphiles capable of transfecting pDNA into different cell lines *in vitro*. To assess the influence of different molecular architectures on gene delivery properties, numerous 1,4-DHP amphiphiles were studied [13–15]. Besides, some of these amphiphilic compounds revealed anticancer activity in HT-1080 and MH-22A cells and antiradical activities (27–40% in DPPH tests) [13, 16]. The remarkable increase of N-H acidity (up to pKa ~7–8) in the DHP cycle is the basis for its buffering activity in these types of gene transfection agents [17].

Recently, it was demonstrated that 4-(N-alkylpyridinium)-1,4-dihydropyridines possess toxicity in Gram-positive and Gram-negative bacteria species and eukaryotic microorganisms [18]. The abovementioned 4-(N-alkylpyridinium)-1,4-dihydropyridines also possess calcium channel-blocking and antioxidant activities [19].

In this work, we chose amphiphilic dihydropyridine derivatives as synthetic lipids and their structural analogues as objects to study pleiotropic activities. The cytotoxic properties of 3 types of amphiphilic compounds in 3 cell lines, namely, normal NIH3T3 (mouse embryonic fibroblast), cancerous HT-1080 (human lung fibrosarcoma), and MH-22A (mouse hepatoma), were evaluated. The studied compounds could be divided into 3 groups according to their structural fragments: (a) 1,4-dihydropyridines containing two cationic moieties as a conjugated cyclic bis(β -carbonylvinyl)amino system and some structurally related compounds; (b) 3,4-dihydro-2(1H)-pyridones containing a cationic moiety as an isomeric 3,4-dihydropyridine structure with an additional

intracyclic carbonyl group—a N- β -carbonylvinylamido system; and (c) 2-amino-3-alkoxycarbonylalkylammonium derivatives as acyclic, open-chain β -aminovinylcarbonyl systems. Lipophilicity of the selected compounds was characterized using logP and molecular topological polar surface area calculations. Evaluation of electrochemical oxidation potentials of the selected compounds was also performed.

2. Materials and Methods

2.1. Chemistry. More detailed descriptions of synthetic procedures and characterization of the original unpublished intermediates and compounds are described in the Supplementary data.

2.1.1. Synthesis of Cationic 1,4-Dihydropyridines 1–26. Briefly, the elaborated synthesis of the cationic 1,4-DHP 1–7, 9–25 involved three sequential steps. The first step was the synthesis of the corresponding 2,6-dimethyl 1,4-DHP derivative in a two-component Hantzsch-type cyclization using 1.0 eq of the corresponding aldehyde, 1.2 eq of an ammonia source, and 2.0 eq of the corresponding acetoacetate for symmetrically substituted 1,4-DHP derivatives or 1 eq of the corresponding acetoacetate and 1.0 eq of the corresponding 3-aminobut-2-enoate for unsymmetrically substituted ones. The second step involved the bromination of the methyl groups of the 2,6-dimethyl-1,4-DHP derivative with N-bromosuccinimide, and the third step was the nucleophilic substitution of bromine of the 2,6-dibromomethylene-1,4-DHP by N-heterocycles or amines yielding the target compounds. The first step for the synthesis of the parent 1,4-dihydropyridine in the case of compound 26 involved the reaction between 1.0 eq of the corresponding aldehyde, 2.4 eq of ammonium acetate, and 4.0 eq of the corresponding acetoacetate. More detailed synthetic procedures and characterization of the original compounds are described in the Supplementary data.

2.1.2. Synthesis of Compound 8 [20]. Briefly, the condensation of ethyl 4-chloroacetoacetate with glyoxylic acid monohydrate in the presence of piperidine/acetate provided (*E,Z*)-2-(2-chloroacetyl)-but-2-enedioic acid 1-ethyl ester, which was used in the next reaction with ethyl 3-amino-4-chlorobut-2-enoate. The obtained 3,5-diethyl 2,6-bis(chloromethyl)-1,4-dihydropyridine-3,4,5-tricarboxylate was esterified with methanol to afford the parent 2,6-dichloromethylene-1,4-DHP. The target compound 8 was obtained *via* nucleophilic substitution of chlorine by pyridine in the presence of potassium iodide.

2.1.3. Synthesis of Cationic Pyridine 27 [14]. Briefly, the corresponding 2,6-dibromomethylene-1,4-DHP was oxidized by HNO₂ followed by nucleophilic substitution of bromine with pyridine to give the target compound 27.

2.1.4. Synthesis of Cationic 3,4-Dihydro-2(1H)-pyridones 28–38. Briefly, 3,4-dihydro-2(1H)-pyridone synthesis employed a four-component reaction using Meldrum's acid by a heterocyclization with the corresponding β -ketoester and the corresponding aldehyde. The obtained 3,4-dihydro-2(1H)-pyridones were treated with bromine giving the respective

6-methylbromides, which in subsequent reaction with pyridine or amine provided the target compounds **28–38**.

2.1.5. Synthesis of 2-Amino-3-alkoxycarbonylalkylammonium Halides 39–48. Briefly, the first step included the transesterification of the commercially available ethyl 4-chloroacetoacetate with a corresponding carbinol without solvent by azeotropic removal of ethanol. The further reaction of the corresponding 4-chloro-3-oxobutanoates with ammonium acetate led to enaminoesters, which were used for quaternization by tertiary amines or heterocycles giving the target compounds **39–48**. Potassium iodide or sodium bromide additives were used for obtaining the corresponding iodides or bromides.

2.2. Experiments for Evaluation of Electrochemical Oxidation Potentials. Cyclic voltammetry experiments were carried out on a PARSTAT 2273 electrochemical system. A stationary glassy carbon disk electrode ($d = 0.5$ mm) served as the working electrode, while the counterelectrode was a Pt wire. The oxidation potentials were measured relative to a Ag/Ag^+ reference electrode. Acetonitrile was dried over P_2O_5 and distilled; the distillate was stored over CaH_2 and redistilled just before use. Recrystallized tetrabutylammonium tetrafluoroborate (TBABF_4) was used as a supporting electrolyte at 0.1 M concentration.

2.3. Determination of LogP and Molecular Topological Polar Surface Area. LogP and Topological Polar Surface Area (TPSA) values were calculated with the Chem3D Ultra 19 program (PerkinElmer Informatics). For logP calculations, the Molecular Networks module was used on single-molecule compounds (salts were taken as cations).

2.4. Cytotoxicity. Cytotoxicity of the compounds was determined on HT-1080 (human lung fibrosarcoma) and MH-22A (mouse hepatoma) cell lines and on the normal NIH3T3 (mouse embryonic fibroblast) cell line. IC_{50} is the compound concentration ($\mu\text{g}/\text{ml}$) at which 50% of the cells die. CV is a triarylmethane dye that can bind to ribose-type molecules such as DNA in nuclei. CV staining can be used to quantify the total DNA of the remaining population and thus is used to determine the number of live cells based on the concentration of the dye which remains after staining. MTT is a standard colorimetric assay used to measure cellular proliferation. Yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan in the mitochondria of living cells.

Reduction takes place only when mitochondrial reductase enzymes are active, and therefore, conversion is directly related to the number of viable cells which can be quantified by the absorbance of the solution (between $\lambda = 500$ and 600 nm) using a spectrophotometer.

2.5. Cell Culture and Measurement of Cell Viability. Tumor cell lines HT-1080 (human connective tissue fibrosarcoma, ATCC® CCL-121™) and MH-22A (mouse hepatocarcinoma, ECACC, cat. Nr. 96121721) were used.

HT-1080 and MH-22A cells were seeded in 96-well plates in Dulbecco's modified Eagle's (DMEM) medium containing 10% fetal bovine serum and 4 mM L-glutamine,

without antibiotics, and cultivated for 72 h by exposure to different concentrations of compounds. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, after incubating with compounds, the culture medium was removed and fresh medium with 0.2 mg/ml MTT was added in each well of the plate. After incubation (3 h, 37°C, 5% CO_2), the medium with MTT was removed, and 200 μl DMSO was added at once to each sample. The samples were tested at 540 nm on a Tecan Infinite M1000 multiplate reader. The IC_{50} was calculated using the program GraphPad Prism® 3.0.

For the CV assay, cells were stained with 0.05% crystal violet (Sigma-Aldrich) in 30% methanol for 20 minutes at room temperature. After incubation, the staining solution was removed. The cells were washed 4 times with water. For dye solubilization, 200 μl of a solubilizing solution (0.1 M citrate buffer, pH 4.2 in 50% ethanol; 1:1 v/v) was added. The absorbance of the solution was measured using a Tecan Infinite M1000 multiplate spectrophotometer at a wavelength of 570 nm [21].

2.6. Basal Cytotoxicity Test. The Neutral Red Uptake (NRU) assay was performed according to the standard protocol of Stokes et al. [22] modified by a NICEATM-ECVAM validation study [23]. The NRU cytotoxicity assay procedure is based on the ability of viable cells to incorporate and bind neutral red, a supravital dye.

Balb/c NIH 3T3 (mouse Swiss albino embryo fibroblast, ATCC® CRL-1658™) cells (9000 cells/well) were placed into 96-well plates for 24 h in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum. Then, the cells were exposed to the test compound over a range of seven concentrations (1000, 316, 100, 31, 10, 3, and 1 $\mu\text{g}/\text{ml}$) for 24 h. Untreated cells were used as a control. After 24 h, the medium was removed from all plates. Then, 150 μl of neutral red solution was added (0.05 mg/ml NR in DMEM 24 h pre-incubated at 37°C and then filtered before use through a 0.22 μm syringe filter). Plates were incubated for 3 h, and then, the cells were washed three times with PBS. The dye within viable cells was released by extraction with a mixture of acetic acid, ethanol, and water (1:50:49). The absorbance of neutral red was measured using a spectrophotometer multiplate reader (Tecan Infinite M1000) at 540 nm. The optical density (OD) was calculated using the following formula: $\text{OD (treated cells)} \times 100 / \text{OD (control cells)}$. The IC_{50} values were calculated using the GraphPad Prism® 3.0 program.

2.7. Estimation of LD_{50} from IC_{50} Values. Data from the *in vitro* tests were used for estimating the starting dose for acute oral systemic toxicity tests in the rodent. The *in vivo* starting dose is an estimated LD_{50} value calculated by inserting the *in vitro* IC_{50} value into a regression formula: $\log \text{LD}_{50} (\text{mM}/\text{kg}) = 0.439 \log \text{IC}_{50} (\text{mM}) + 0.621$ [23–25]. The value is recalculated to mg/kg, and compounds are evaluated in accordance with 4 toxicity categories [26]: category 1— $\text{LD}_{50} \leq 5$ mg/kg (highly toxic); category 2— $5 < \text{LD}_{50} \leq 50$ mg/kg (moderately toxic); category 3— $50 < \text{LD}_{50} \leq 300$ mg/kg (slightly toxic); and

category $4-300 < LD_{50} \leq 2000$ mg/kg (practically non-toxic). Using an alternative *in vitro* method allows the comparison between possibly toxic new compounds and selecting compounds for further study vastly reducing the number of animal experiments.

3. Results and Discussion

3.1. Structures of Compounds. The studied compounds could be divided into 3 groups considering structure fragments:

- (1) *1st group*: twenty-five representatives of two cationic moieties containing 1,4-dihydropyridines (compounds **1–25** in Table 1) and, additionally, four cationic moieties containing 1,4-dihydropyridine (compound **26** in Table 1) and one as an oxidized form—two cationic moieties containing pyridine (compound **27** in Table 1)
- (2) *2nd group*: eleven representatives of 3,4-dihydro-2(1H)-pyridones containing a cationic moiety as an isomeric 3,4-dihydropyridine structure with an additional intracyclic carbonyl group, i.e., the N- β -carbonylvinylamido system (compounds **28–38** in Table 2)
- (3) *3rd group*: ten representatives of cationic 2-amino-3-alkoxycarbonylalkylammonium derivatives as acyclic, open-chain analogues of cyclic 1,4-DHPs (compounds **39–48** in Table 3)

These three groups were selected after analysis of their structure-activity relationships as synthetic lipid-like amphiphilic compounds. Previously, the 3rd group representatives—(2-amino-3-alkoxycarbonylalkyl)trialkylammonium halides—were synthesized, and their ribonucleic acid (RNS) transfection activity was demonstrated [27]. This group covers synthetic lipid-like compounds on the basis of a β -aminovinylcarbonyl (AVC) moiety: a conjugated pentade system connected with a lipophilic carbon atom chain and a cationic part (alkylammonium or pyridinium type). These compounds are proposed as an open-chain system compared to the cationic 1,4-DHP or pyridone heterocyclic systems. Structurally, the 2nd group molecules—the cationic pyridone derivatives—are heterocycles which comprise a cyclic AVC system and an additional intracyclic carbonyl group and an extracyclic cation. Previously, the 1st group representatives—1,4-DHP derivatives containing pyridinium moieties—were presented as promising tools for delivery of DNA into target cells [13, 14, 20, 28]. It is noteworthy that, due to cross-conjugation of two AVC systems of the 1,4-DHP cycle, its N-H group is influenced by two electron-withdrawing vinylcarbonyls.

3.2. Synthesis of the Amphiphiles. Synthesis of the amphiphiles was performed according to Schemes 1–4.

Previously, 3,5-bis(dodecyloxycarbonyl)-1,4-DHPs containing cationic moieties were elaborated as synthetic lipid-like compounds having promising gene delivery properties for DNA transfection; the basic structure-activity relationships have been verified for the cationic 1,4-DHP derivatives

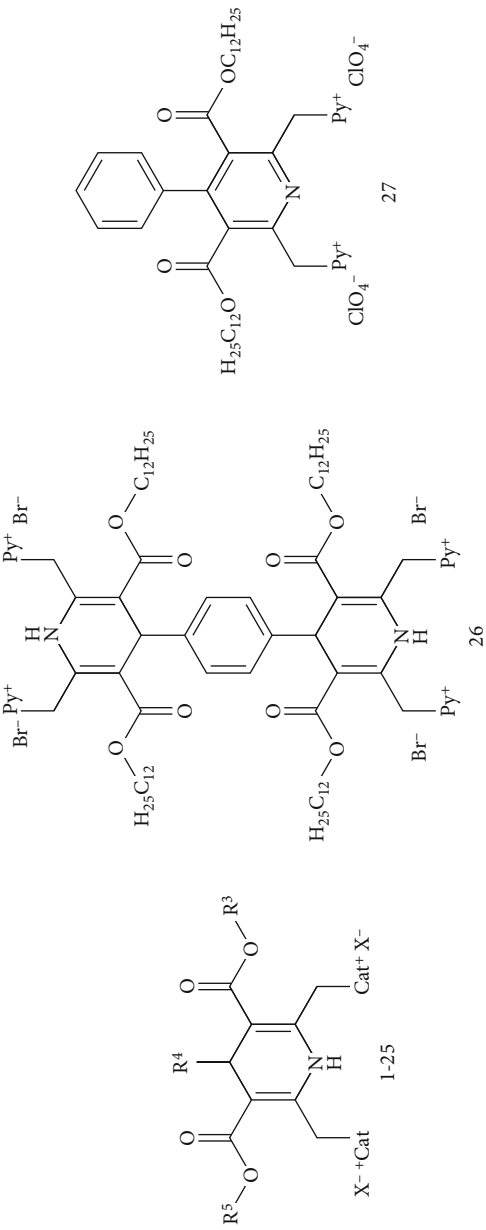
as gene delivery systems, and it has been shown that the molecular structure affected their self-assembling properties, pDNA-binding ability, and properties of the formed 1,4-DHP amphiphile-pDNA complexes [13, 15, 28]. The synthetic procedure for the amphiphilic compounds **1–7**, **9–22**, and **26** is displayed in Scheme 1 via a multistep sequence. The first step was a Hantzsch synthesis of the parent compound 3,5-bis(alkoxycarbonyl)-2,6-dimethyl-4-phenyl-1,4-dihydropyridine. The second step was bromination of the 2,6-methyl groups with N-bromosuccinimide (NBS) [34] resulting in the second parent compound 2,6-di(bromomethyl)-3,5-bis(alkoxycarbonyl)-4-phenyl-1,4-dihydropyridine. The final step was nucleophilic substitution of bromine in the 2,6-di(bromomethyl)-1,4-DHP by N-heterocycles or amines, which is facile and generally takes place in good yields.

Additionally, for studies of targeted changes in the structure of corresponding dicationic compound **10**, which was found to be more active for DNA delivery among the tested 1,4-DHP amphiphiles [13, 15, 28], 1,1',1'',1'''-((1,4-phenylenebis(3,5-bis((dodecyloxy)carbonyl)-1,4-dihydropyridine-4,2,6-triyl))tetrakis(methylene))tetrakis(pyridin-1-ium) tetrabromide (**26**) was synthesized as a double or a “dimeric” molecule of compound **10** and 1,1'-((3,5-bis(dodecyloxycarbonyl)-4-phenylpyridine-2,6-diyl)bis(methylene))-bis(pyridin-1-ium)dibromide (**27**) was prepared in accordance with what was reported in [14] as the oxidized form of compound **10**. For evaluation of the anion influence on cytotoxicity, 1,1'-((3,5-bis((dodecyloxy)carbonyl)-4-phenyl-1,4-dihydropyridine-2,6-diyl)bis(methylene))bis(pyridin-1-ium) ditetrafluoroborate (**11**) was synthesized from compound **10** after treating with NH_4BF_4 .

The unsymmetrical 1,4-DHP amphiphile **23** was designed for analysis of the influence of mixed perfluorinated and alkyl ester moiety substituents on the properties of amphiphiles. The 1,4-DHP **23** molecule contains only one of the two esters as a perfluorinated ester moiety while the other is an alkyl ester—the same as previous compounds. The synthetic procedure (Scheme 2) includes a reaction between an enamine and benzylidene in diglyme with inclusion of n-butylpyridinium chloride as a phase transfer catalyst (PTC) [35] to affording the parent dodecyl 5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,12,12-heptafluorododecyl 1,4-dihydro-2,6-dimethyl-4-phenylpyridine-3,5-dicarboxylate in 43% yield. Without the PTC, the yield was only 25%. Subsequent bromination with NBS formed the corresponding 2,6-dibromomethylene DHP which was used without further purification in nucleophilic substitution with pyridine yielding the unsymmetrical 1,4-DHP **23**.

The 3,4-dihydro-2(1H)-pyridones (DHPDO) possess various pharmacological properties as α_1 adrenergic receptor antagonists [36], Rho-kinase inhibitors [37], P_2X_7 receptor antagonists [38], or G-protein-coupled kinase receptor antagonists [39]. Synthesis of 3,4-dihydro-2(1H)-pyridone amphiphiles **28–38** was performed according to Scheme 3. In this case, Meldrum's acid was used as the second dicarbonyl component in a Hantzsch-like reaction with heterocyclization, with a corresponding β -ketoester and a corresponding aldehyde in the presence of ammonium acetate in refluxing glacial

TABLE 1: Structure, cytotoxicity, and calculated basal toxicity of 1,4-dihydropyridines containing cationic moieties.



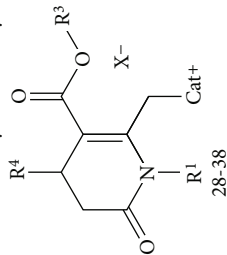
| Comp. | Cat ⁺ | R ³ | R ⁴ | R ⁵ | X ⁻ | HT-1080 | | MH-22A | | NIH3T3 | | Ref. |
|-------|----------------------------|--|---|--|------------------|-----------------------|------------------------|-----------------------|------------------------|-----------------------|--------------------------|----------|
| | | | | | | IC ₅₀ (CV) | IC ₅₀ (MTT) | IC ₅₀ (CV) | IC ₅₀ (MTT) | IC ₅₀ (NR) | LD ₅₀ (mg/kg) | |
| 1 | Py | C ₂ H ₅ | Ph | C ₂ H ₅ | Br | * | * | * | * | * | >2000 | [29] |
| 2 | Py-Me-4 | C ₂ H ₅ | Ph | C ₂ H ₅ | Br | * | >740 | * | * | * | >3360 | [30] |
| 3 | Py-NH ₂ -4 | C ₂ H ₅ | Ph | C ₂ H ₅ | Br | nt | 441 ± 46 | nt | >740 | * | >3360 | Suppl. |
| 4 | Py-NMe ₂ -4 | C ₂ H ₅ | Ph | C ₂ H ₅ | Br | nt | 324 ± 29 | nt | 608 ± 49 | * | >3500 | Suppl. |
| 5 | Py-Me-3 | C ₂ H ₅ | Ph | C ₂ H ₅ | Br | nt | >740 | nt | * | * | >3360 | Suppl. |
| 6 | Py | C ₂ H ₅ | C ₆ H ₄ -CF ₃ -2 | C ₂ H ₅ | Br | * | * | * | * | * | >2000 | [20] |
| 7 | Py | C ₂ H ₄ OC ₃ H ₇ | C ₆ H ₄ -OCHF ₂ -2 | C ₂ H ₄ OC ₃ H ₇ | Br | * | * | * | * | * | >2000 | [20] |
| 8 | Py | C ₂ H ₅ | COOCH ₃ | C ₂ H ₅ | I | * | * | * | * | * | >2000 | [20] |
| 9 | N-Me-morph | CH ₃ | C ₆ H ₄ -OCHF ₂ -2 | CH ₃ | ClO ₄ | * | * | * | * | * | >2000 | [31] |
| 10 | Py | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Br | 3 ± 0.5 | 3 ± 0.3 | 6 ± 1 | 3 ± 0.8 | 100 ± 6 | 1482 | [13, 28] |
| 11 | Py | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | BF ₄ | 31 ± 4 | 28 ± 9 | 30 ± 6 | 30 ± 9 | 47 ± 8 | 1053 | Suppl. |
| 12 | Py-Me-4 | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Br | 10 ± 2 | 5 ± 1 | 40 ± 2 | 29 ± 2 | 79 ± 11 | 1431 | [13] |
| 13 | Py-NMe ₂ -4 | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Br | 10 ± 1 | 3 ± 0.6 | 6 ± 2 | 10 ± 3 | 119 ± 13 | 1706 | [13] |
| 14 | Py-C(=O)CH ₃ -3 | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Br | 3 ± 0.4 | 3 ± 0.3 | 100 ± 13 | 49 ± 9 | 922 ± 24 | 4040 | [13] |
| 15 | N-Me-morph | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | ClO ₄ | 13 ± 3 | 12 ± 3 | 34 ± 11 | 27 ± 9 | 35 ± 13 | 979 | [13] |
| 16 | Pyr | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Br | * | * | * | * | * | >2000 | [13] |
| 17 | NMe ₃ CyHex | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Br | 35 ± 9 | 23 ± 3 | 54 ± 16 | 35 ± 9 | 59 ± 12 | 1274 | Suppl. |

TABLE 1: Continued.

| Comp. | Cat ⁺ | R ³ | R ⁴ | R ⁵ | X ⁻ | HT-1080 | | MH-22A | | NIH3T3 | | Ref. |
|-------|---|--|-------------------------------------|--|------------------|----------------------------------|-----------------------------------|----------------------------------|-----------------------------------|----------------------------------|-----------------------------|-------|
| | | | | | | IC ₅₀ (CV) (μg/ml) | IC ₅₀ (MTT) (μg/ml) | IC ₅₀ (CV) (μg/ml) | IC ₅₀ (MTT) (μg/ml) | IC ₅₀ (NR) (μg/ml) | LD ₅₀ (mg/kg) | |
| 18 | Py | C ₁₂ H ₂₅ | C ₆ H ₄ -OH-4 | C ₁₂ H ₂₅ | Br | 4.3 ± 0.6 | 10 ± 2 | 3.4 ± 0.6 | 16 ± 2 | 95 ± 7 | 1479 | Suppl |
| 19 | Py-CH ₃ -4 | C ₁₂ H ₂₅ | C ₆ H ₄ -OH-4 | C ₁₂ H ₂₅ | Br | 32 ± 8 | 22 ± 5 | 21 ± 3 | 30 ± 8 | 44 ± 11 | 1087 | Suppl |
| 20 | N-Me ₂ C ₁₂ H ₂₅ | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Br | 3 ± 0.3 | 3 ± 0.4 | 10 ± 2 | 10 ± 2 | 19 ± 6 | 836 | Suppl |
| 21 | Py-CF ₃ -4 | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Br | 2 ± 0.3 | 4 ± 0.5 | 49 ± 8 | 19 ± 6 | 12 ± 4 | 619 | [32] |
| 22 | Py | C ₁₂ H ₂₄ CF ₃ | Ph | C ₁₂ H ₂₄ CF ₃ | ClO ₄ | 18 ± 5 | 10 ± 2 | 10 ± 1 | 19 ± 5 | 16 ± 3 | 771 | [32] |
| 23 | Py | C ₁₂ H ₂₅ | Ph | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Br | 50 ± 6 | 47 ± 8 | 47 ± 2 | 75 ± 11 | 477 ± 25 | 3448 | Suppl |
| 24 | Py | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Ph | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Br | 100 ± 11 | 100 ± 9 | * | * | * | >2000 | Suppl |
| 25 | Py | (CH ₂) ₁₁ (CF ₂) ₇ CF ₃ | Ph | (CH ₂) ₁₁ (CF ₂) ₇ CF ₃ | Br | * | * | * | * | * | >2000 | Suppl |
| 26 | | "Dimeric" form of comp. 10 | | | Br | 12 ± 4 | 14 ± 2 | 27 ± 6 | 21 ± 4 | 717 ± 56 | 5164 | Suppl |
| 27 | | Oxidated form of comp. 10 | | | ClO ₄ | 3.2 ± 0.8 | 3.1 ± 0.5 | 3.0 ± 0.3 | 3.3 ± 0.4 | 900 ± 42 | 3946 | [14] |

nt: not tested; *: not detected.

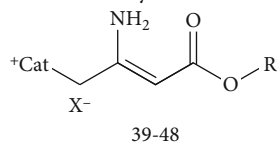
TABLE 2: Structure, cytotoxicity, and calculated basal toxicity of 3,4-dihydro-2(1*H*)-pyridones containing cationic moiety.



| Comp. | R ¹ | Cat ⁺ | R ³ | R ⁴ | X ⁻ | HT-1080 | | | MH-22A | | NIH3T3 | | Ref. |
|-------|-------------------|---|---|---|------------------|----------------------------------|-----------------------------------|----------------------------------|-----------------------------------|----------------------------------|-----------------------------|-------|------|
| | | | | | | IC ₅₀ (CV) (μg/ml) | IC ₅₀ (MTT) (μg/ml) | IC ₅₀ (CV) (μg/ml) | IC ₅₀ (MTT) (μg/ml) | IC ₅₀ (NR) (μg/ml) | LD ₅₀ (mg/kg) | | |
| 28 | H | Py | CH ₃ | C ₆ H ₄ -OCHF ₂ -2 | Br | * | * | >100 | * | 1132 ± 85 | >2000 | Suppl | |
| 29 | PhCH ₂ | Py | CH ₃ | H | Br | * | * | * | * | 972 ± 27 | >2000 | Suppl | |
| 30 | H | PPh ₃ | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Ph | Br | 10 ± 2 | 9 ± 2 | 30 ± 11 | 39 ± 5 | 127 ± 11 | 1779 | [33] | |
| 31 | H | Py | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Ph | Br | 3 ± 0.3 | 3 ± 0.2 | 3 ± 1 | 3 ± 0.6 | 15 ± 2 | 604 | [33] | |
| 32 | H | Py | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Ph | ClO ₄ | 3 ± 0.3 | 3 ± 0.5 | 2 ± 0.3 | 3 ± 0.2 | 15 ± 3 | 618 | Suppl | |
| 33 | H | N-Me ₂ C ₁₂ H ₂₅ | CH ₃ | Ph | Br | 2 ± 0.4 | 1 ± 0.2 | 2 ± 0.1 | 2 ± 0.6 | 4 ± 1 | 269 | Suppl | |
| 34 | H | N-Me ₂ C ₁₂ H ₂₅ | C ₁₂ H ₂₅ | Ph | Br | 2 ± 0.2 | 2 ± 0.6 | 3 ± 0.8 | <1 | 6 ± 2 | 346 | Suppl | |
| 35 | H | N-Me ₂ C ₁₂ H ₂₅ | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Ph | Br | 3 ± 0.3 | 30 ± 11 | 26 ± 6 | 30 ± 8 | 27 ± 9 | 898 | Suppl | |
| 36 | H | N-Me ₂ C ₁₂ H ₂₅ | CH ₃ | H | Br | 2 ± 0.2 | 1 ± 0.2 | 18 ± 3 | 16 ± 3 | 63 ± 11 | 831 | Suppl | |
| 37 | H | N-Me ₂ C ₁₂ H ₂₅ | C ₁₂ H ₂₅ | H | Br | 2 ± 0.5 | 3 ± 0.4 | 3 ± 0.6 | 1 ± 0.2 | 7 ± 1 | 369 | Suppl | |
| 38 | H | N-Me ₂ C ₁₂ H ₂₅ | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | H | Br | 3 ± 0.2 | 2 ± 0.2 | 4 ± 0.8 | <1 | 11 ± 3 | 553 | Suppl | |

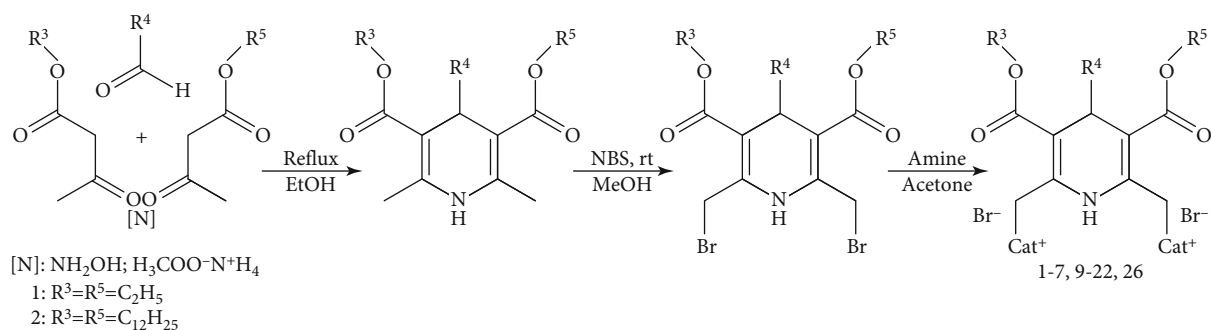
∗: not detected.

TABLE 3: Structures, cytotoxicity, and calculated toxicity of 2-amino-3-alkoxycarbonylalkylammonium halides.



| Comp. | Cat ⁺ | R | X ⁻ | HT-1080 | | MH-22A | | NIH3T3 | | Ref. |
|-------|---|---------------------------------|----------------|----------------------------------|-----------------------------------|----------------------------------|-----------------------------------|----------------------------------|-----------------------------|-------|
| | | | | IC ₅₀ (CV) (μg/ml) | IC ₅₀ (MTT) (μg/ml) | IC ₅₀ (CV) (μg/ml) | IC ₅₀ (MTT) (μg/ml) | IC ₅₀ (NR) (μg/ml) | LD ₅₀ (mg/kg) | |
| 39 | N-Me ₂ CyHex | C ₁₆ H ₃₃ | Cl | 0.35 ± 0.06 | 0.5 ± 0.1 | 1 ± 0.6 | 0.2 ± 0.06 | 0.7 ± 0.1 | 97 | Suppl |
| 40 | N-Me(CH ₂) ₄ | C ₁₆ H ₃₃ | Cl | <1 | <1 | 1 ± 0.2 | <1 | 8 ± 1 | 312 | Suppl |
| 41 | N-Me ₂ (CH ₂) ₂ NMe ₂ | C ₁₆ H ₃₃ | Cl | 2 ± 0.3 | 2 ± 0.6 | 1 ± 0.3 | 1 ± 0.2 | 5 ± 1 | 286 | Suppl |
| 42 | N-Me(CH ₂) ₂ N(CH ₂) ₂ Me | C ₁₆ H ₃₃ | Cl | 2 ± 0.4 | 2 ± 0.2 | 1 ± 0.1 | 2 ± 0.3 | 4 ± 1 | 237 | Suppl |
| 43 | N-Me ₂ C ₆ H ₁₃ | C ₁₆ H ₃₃ | Cl | 3 ± 0.6 | 2 ± 0.4 | 2 ± 0.2 | 1 ± 0.2 | 21 ± 6 | 538 | Suppl |
| 44 | N-Me ₂ C ₁₂ H ₂₅ | C ₁₆ H ₃₃ | Br | 3 ± 0.4 | 3 ± 0.3 | 25 ± 3 | 28 ± 6 | 31 ± 8 | 697 | Suppl |
| 45 | N-Me ₂ C ₁₂ H ₂₅ | C ₁₆ H ₃₃ | Cl | nt | nt | nt | nt | 25 ± 9 | 573 | Suppl |
| 46 | N-Me ₂ C ₁₂ H ₂₅ | C ₁₆ H ₃₃ | I | nt | nt | nt | nt | 34 ± 8 | 637 | Suppl |
| 47 | Py | C ₁₂ H ₂₅ | I | 3 ± 0.4 | nt | 2 ± 0.1 | nt | 14 ± 9 | 403 | Suppl |
| 48 | N-Me ₂ C ₁₂ H ₂₅ | C ₁₀ H ₂₁ | Cl | 3 ± 0.4 | nt | 2 ± 0.2 | nt | 17 ± 3 | 485 | Suppl |

nt: not tested.



SCHEME 1: Synthesis of 1,4-dihydropyridine (1,4-DHP) amphiphiles 1–7, 9–22, and 26.

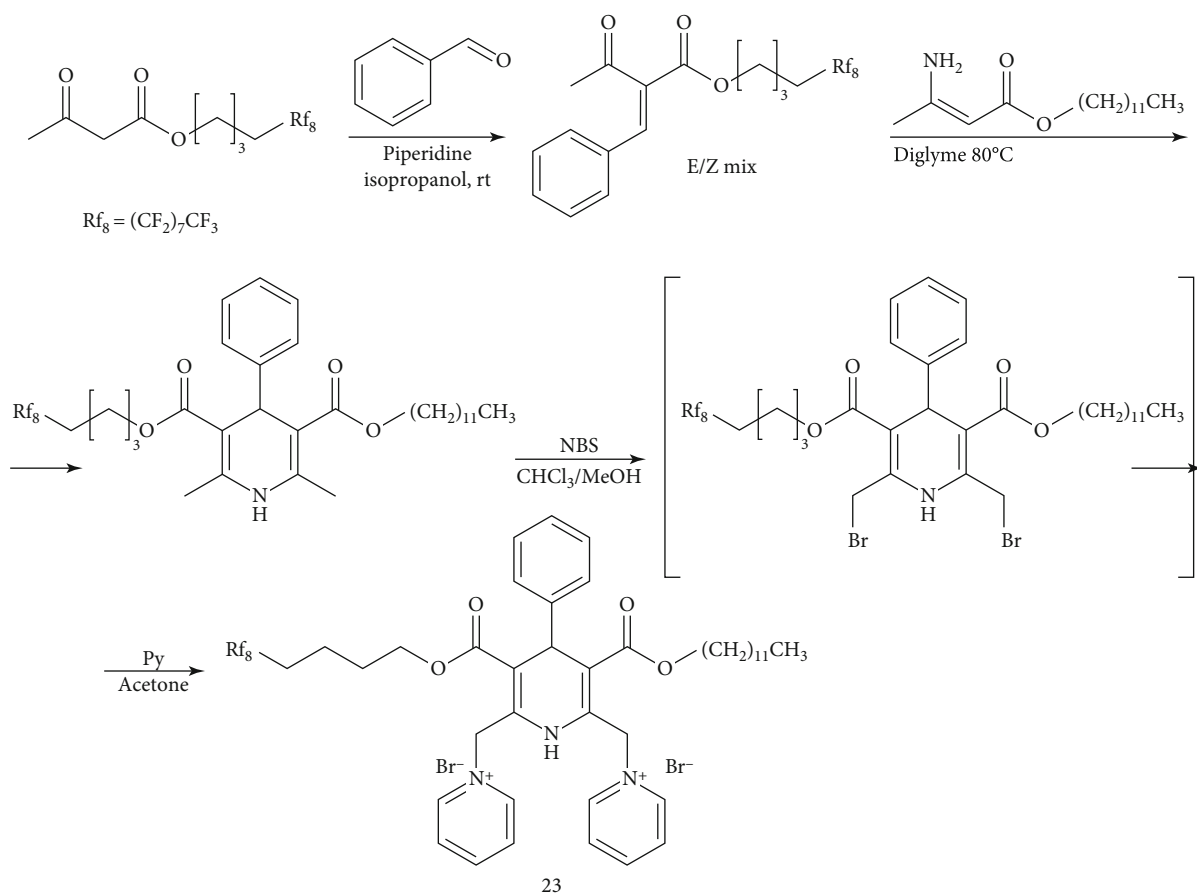
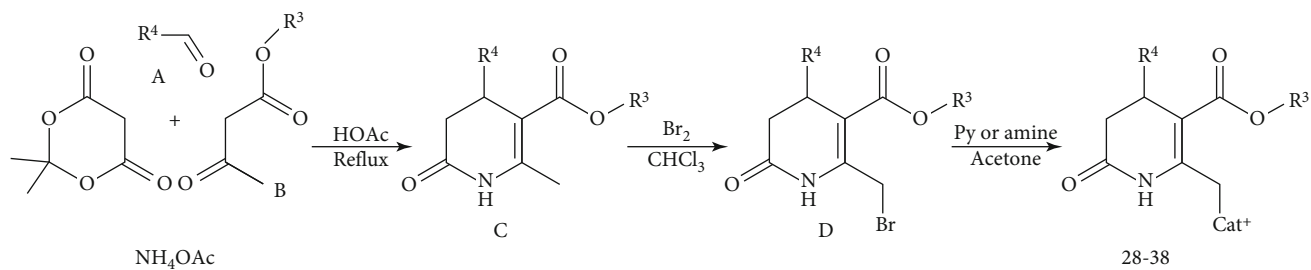
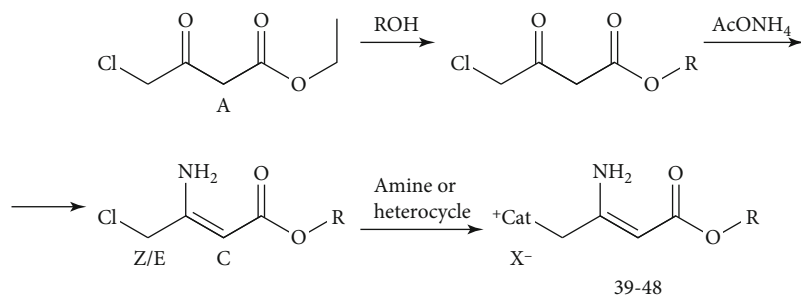
acetic acid [40]. The parent DHPDO solution in chloroform was treated with bromine affording the respective 6-bromo-methyl-DHPDO, which on subsequent reaction with pyridine or N,N-dimethyldodecyl-1-amine in dry acetone yielded the corresponding amphiphilic DHPDO derivatives **28–38**.

Typically, enaminones have been studied and used as precursors and synthons for the synthesis of novel heterocyclic systems: pyridines, pyrroles, pyrimidines, dihydropyridines, etc. [41]. Therefore, up to now, most of the research in the field of enamines have been devoted to the elaboration of new strategies and synthetic methods, but lack biological studies [42]. 2-Amino-3-alkoxycarbonylalkylammonium halides with long alkyl chains have appeared as a new class of enaminoesters and were elaborated as a transfection agent for RNS transfection [27]. Also, structure analogues—alkyl acyl carnitine esters—were synthesized and characterized as biocompatible cationic lipids for use in gene delivery [43]. Cationic enaminoesters are stable solid compounds with remarkable solubility in water. The synthetic procedure for 2-amino-3-alkoxycarbonylalkylammonium halides **39–48** is

provided in Scheme 4. Briefly, the first step included the transesterification of commercially available ethyl 4-chloroacetoacetate with the corresponding carbinol without solvent by azeotropic removal of ethanol. Further reaction of the corresponding 4-chloro-3-oxobutanoates with ammonium acetate led to the formation of enaminoesters, which were used for quaternization with tertiary amines or heterocycles by extended heating in a dry solvent. Potassium iodide or sodium bromide additives were used for obtaining the corresponding iodides or bromides.

The perchlorates of amphiphiles **9**, **15**, **22**, **27**, and **32** were obtained from the corresponding bromides by treating with excess of conc. HClO₄ according to the procedure elaborated by Turovska et al. [44]. In some cases, the perchlorates were used for obtaining solid salts while the corresponding bromides existed as oils, and it was not possible to isolate them from the reaction mixture.

The full description of the synthesis and characterization of the original compounds in detail are given in the Supplementary data. Purity of the studied compounds was at least

SCHEME 2: Synthesis of unsymmetrical 1,4-DHP amphiphile **23**.SCHEME 3: Synthesis of 3,4-dihydro-2(1H)-pyridone (DHPDO) amphiphiles **28-38**.SCHEME 4: Synthesis of 2-amino-3-alkoxycarbonylalkylammonium halides **39-48**.

97% according to high-performance liquid chromatography (HPLC) data.

3.3. Estimation of LD_{50} from IC_{50} Values. It has been proposed that the equation from the correlation of IC_{50} (the concentration of a substance that causes 50% toxicity *in vitro*) could be applied to estimate unknown LD_{50} values for a new compound from IC_{50} values measured as basal cytotoxicity *in vitro*. This estimated LD_{50} gives prior information regarding compound properties and would be used to select promising compounds and a starting dose for *in vivo* experiments. The evaluation of cytotoxicity of the abovementioned 3 types of amphiphilic compounds *in vitro* was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet (CV) assays on two monolayer tumor cell lines, namely, HT-1080 (human fibrosarcoma) and MH-22A (mouse hepatoma). Additionally, the compound influence on “normal” mouse fibroblasts (NIH3T3) was estimated for the studies of structure-activity relationships and exploration of the effect of substituents. The results are presented in Tables 1–3. Studies of cytotoxicity of the amphiphilic self-assembling compounds revealed certain regularities.

3.4. Analysis of Structure-Activity Relationships

3.4.1. Two Cationic Moieties Containing 1,4-DHP Amphiphiles (Table 1)

(1) Modification of Ester Alkyl Groups. Compounds possessing short alkyl chains in the ester groups at positions 3 and 5 of the dihydropyridine ring and organic heterocyclic cations in the methylene groups in positions 2 and 6 had very low cytotoxicity in cancer cell lines HT-1080 and MH-22A and high calculated LD_{50} values (comp. 1–9). It means that the compounds were almost inert to the noncancerous cell line NIH3T3 and cancer cell lines HT-1080 and MH-22A. This refers to several groups of the mentioned type of compounds possessing short alkyls in 3,5-ester substituents (methyl, ethyl, and also the more elongated propoxyethyl groups): comp. 9, 1, 6, 8, and 7. Additionally, 3,5-bis(diethyloxycarbonyl)-1,4-DHP amphiphiles comprising substituted pyridinium moieties in positions 2 and 6 (compounds 2–5) have moderate cytotoxicity, around 3300 mg/kg. In most cases, for the 3,5-bis(diethyloxycarbonyl)-1,4-DHP compounds, it was not possible to calculate IC_{50} values for the cancer cell lines HT-1080 and MH-22A due to rather low cytotoxicities; calculated LD_{50} values of compounds 1–9 were >2000 mg/kg (for NIH3T3 cells).

On the contrary, analogues comprising dodecyl chains in the ester groups at positions 3 and 5 and pyridinium moieties in positions 2 and 6 of the dihydropyridine ring (see comp. 10 versus comp. 1, comp. 12 versus comp. 2, comp. 13 versus comp. 4) showed significant cytotoxicity towards cancer cell lines HT-1080 and MH-22A and still very low cytotoxicity in noncancerous NIH3T3 cells. For example, LD_{50} values for compounds 10, 12, and 13 were 1482, 1431, and 1706 mg/kg, respectively, while cytotoxicity towards HT-1080 cells was 3–10 μ g/ml and towards

MH-22A cells was 3–40 μ g/ml. In principle, the obtained data coincide with observations on the impact of dihydropyridine on cell growth, where it was concluded that a long alkyl chain containing 1,4-DHP amphiphiles show promising dual activity—proliferation inhibition on cancer cell lines and proliferation stimulating effect on normal cell lines [31]. It should be noted that the 3,5-bis(dodecyloxycarbonyl)-1,4-DHP amphiphile 14 with an electron-withdrawing acetyl group in the pyridinium moieties in positions 2 and 6 of the 1,4-DHP ring was practically nontoxic on noncancerous NIH3T3 cells and demonstrated selective cytotoxicity toward cancer cells and pronounced cytotoxicity of around 3 μ g/ml on HT-1080 cells and of 50–100 μ g/ml on MH-22A cells.

Substitution of fluorine for hydrogen atoms in alkyl chains of the 1,4-DHP amphiphiles decreased cytotoxicity of the abovementioned compounds. Thus, substitution of the 3,5-dodecyloxycarbonyl alkyl chain's most distant hydrogen atom by trifluoromethyl groups (comp. 22 versus comp. 10) leads to lower cytotoxicity to cancer cell lines HT-1080 (10–18 μ g/ml versus 3 μ g/ml) and MH-22A (10–19 μ g/ml versus 3–6 μ g/ml) and to higher cytotoxicity to noncancerous cell line NIH3T3 (771 mg/kg versus 1482 mg/kg). In this case, the influence of the anion was not taken into account, but that also could give an effect. Substitution of seven methylene groups of a 3-dodecyloxy moiety by seven difluoromethylene groups leads to the formation of an unsymmetrical 1,4-DHP amphiphile 23, which has lower cytotoxicity to the studied cancer and normal cell lines (comp. 23 versus comp. 10)—a cytotoxicity of around 47–75 μ g/ml versus 3–6 μ g/ml on cancer cell lines and LD_{50} 3448 mg/kg versus 1482 mg/kg, respectively. Further substitution of the next seven methylene groups by seven difluoromethylene groups (comp. 24) leads to subsequent diminishing of cytotoxicity to cancer cell lines (around 100 μ g/ml) and also low calculated toxicity to noncancerous cell line NIH3T3 (>2000 mg/kg). In the case of the more extended partially fluorinated 3,5-heptadecafluorononadecyl chains (comp. 25), the compounds revealed undetectable toxicity to the two cancer cell lines and also low toxicity to the noncancerous cell line NIH3T3. So, from the obtained results (Table 1), it is evident that the perfluorinated 1,4-DHP amphiphiles (compounds 23–25) are nontoxic in the tested cell lines.

(2) Modification of Substituents at Position 4 of the 1,4-DHP Ring. Variations of several types of substituents in position 4 of the DHP ring of 3,5-bis(diethyloxycarbonyl)-1,4-DHPs were performed. Compounds with phenyl (comp. 1–5), substituted phenyl (2-difluoromethylphenyl (comp. 7 and 9), trifluoromethyl (comp. 6)), and ethoxycarbonyl (comp. 8) substituents were obtained, but no significant influence on the cytotoxicity of the tested 1,4-DHP amphiphiles was observed. Introducing an HO-substituent in a phenyl moiety in position 4 of the 1,4-DHP ring gives comp. 18 and 19 which are 4-(4'-hydroxyphenyl) analogues of corresponding 4-phenyl-DHPs 10 and 12, respectively. It was demonstrated that the introduction of an HO-substituent does not give a strong influence on the cytotoxicity of the compounds.

(3) *Modification of the Cationic Moieties.* Modification of the cationic moieties in positions 2 and 6 of the 1,4-DHP ring (in the case of 3,5-dodecyloxycarbonyl substituents) may result in substantially different toxicological properties. The insertion of substituents in the pyridinium ring leads to some quantitative modifications of cytotoxicity—mainly to slightly diminished cytotoxicity in the case of 4-methyl and 4-dimethylamino substituents (comp. **12** and comp. **13**)—while introducing a 3-acyl substituent (comp. **14** versus comp. **10**) in the pyridinium moiety did not give any influence on the cytotoxicity in HT-1080 cells (in both cases around 3 $\mu\text{g/ml}$), but decreased cytotoxicity in MH-22A cells (50–100 $\mu\text{g/ml}$ and 3–6 $\mu\text{g/ml}$, respectively) and also decreased cytotoxicity in noncancerous cell line NIH3T3 (4040 mg/kg versus 1482 mg/kg). Comparison of the cytotoxicity of the compounds with 4-methylpyridinium and 4-trifluoromethyl pyridinium moieties (comp. **12** versus comp. **21**) showed that there are no significant changes of cytotoxicity in cancer HT-1080 and MH-22A cells (5–10 $\mu\text{g/ml}$ versus 2–4 $\mu\text{g/ml}$, and 29–40 $\mu\text{g/ml}$ versus 19–49 $\mu\text{g/ml}$, respectively) but a twofold increase of cytotoxicity in noncancerous NIH3T3 cells (1431 mg/kg versus 619 mg/kg). Introduction of a pyrazinium moiety instead of a pyridinium moiety (comp. **16** versus comp. **10**) gave a compound which was practically inert to the studied cancer cell lines HT-1080 and MH-22A and was also nontoxic to normal NIH3T3 cells. Exchange of the heteroaromatic pyridinium moieties to saturated heterocyclic moieties—N-methylmorpholinium fragments (comp. **15**)—led to slightly lower anticancer activity of the compound towards the abovementioned cancer cell lines and comparatively higher cytotoxicity to NIH3T3 cells (979 mg/kg). Also, in this case, the influence of the anion was not taken into account, but it may give an effect (ClO_4^- instead of Br^-). Introducing N,N-dimethylcyclohexylammonium moieties as the cationic part of the amphiphile gave compound **17** with less cytotoxicity (23–54 $\mu\text{g/ml}$) on both tested cancer cell lines and an LD_{50} value of 1274 mg/kg, while the LD_{50} value of compound **18** with the introduced aliphatic N,N-dimethyl-N-dodecylammonium moieties was 836 mg/kg. It was shown that compound **18** with aliphatic ammonium fragments was more cytotoxic. The obtained data is in agreement with conclusions by Lv et al. that among the synthetic cationic delivery systems, quaternary ammonium surfactants are more toxic than their analogues with the cationic charge delocalized in a heterocyclic system [45].

(4) *Change of the Anions.* Insertions of the BF_4^- anion instead of the usual Br^- anion (comp. **11** versus comp. **10**) lead to an increase in basal toxicity—1053 and 1482 mg/kg, respectively—but a decrease in cytotoxicity on HT-1080 (around 30 $\mu\text{g/ml}$ and 3 $\mu\text{g/ml}$, respectively) and MH-22A (30 $\mu\text{g/ml}$ and 3–6 $\mu\text{g/ml}$, respectively) cell lines.

(5) *Change of Dehydrogenation Degree.* Lipid-like pyridine derivative **27**, as the oxidized form of compound **10**, demonstrated very close cytotoxicity data to the corresponding dihydro compound **10**, in all cases around 3 $\mu\text{g/ml}$, while

basal toxicity on noncancerous cell line NIH3T3 is significantly diminished: comp. **27** is not harmful at all (LD_{50} is 3948 mg/kg). In this case also, the influence of the anion was not taken into account, but it may give an effect (ClO_4^- instead of Br^-).

(6) *The Duplication of Moieties.* Synthetic lipid-like compound **26** was obtained as a “dimeric” form of compound **10**, which was proposed as a promising DNA delivery agent. Cytotoxicity data of amphiphiles (comp. **26** versus comp. **10**) demonstrated that this structural modification slightly decreased cytotoxicity of the target compound **26** in both tested cancer HT-1080 and MH-22A cells (12–27 $\mu\text{g/ml}$ versus 3–6 $\mu\text{g/ml}$, respectively) and also considerably decreased the LD_{50} value—5164 mg/kg versus 1482 mg/kg.

3.4.2. Cationic Moiety Containing 3,4-Dihydro-2(1H)-pyridones (Table 2)

(1) *Modification of Ester Alkyl Group.* Same as in the case of 1,4-DHP amphiphiles also, pyridones with a short alkyl chain—the methyl group in the ester moiety and pyridinium in the cationic part of the molecule (comp. **28** and **29**)—were inert to tested cancer cells HT-1080 and MH-22A and demonstrated high calculated LD_{50} values > 2000 mg/kg. In the case of comp. **29**, the influence of the N-substituent in the pyridone and, in the case of comp. **28**, the influence of substituent in the phenyl moiety at position 4 of the pyridone cycle were not taken into account. Two other methyl esters—comp. **33** and **36**—demonstrated significant cytotoxicity on all the tested cell lines. This could be explained by the influence of the cationic moiety in the compound.

Substitution of fluorine for hydrogen atoms in the alkyl chain of the pyridone amphiphiles did not give a strong influence on the cytotoxicity of the compounds, and it seems that this also was dependent on the cationic moiety and other substituents of the compound. So, in the case of 4-unsubstituted pyridones with the N,N-dimethylcyclohexylammonium moiety, comp. **37** with hydrogen atoms in the ester moiety and comp. **38** with fluorine atoms in the ester moiety demonstrated similar cytotoxicity in all of the tested cell lines, while for cytotoxicity of 4-phenyl pyridones with N,N-dimethylcyclohexylammonium moiety, comp. **34** with hydrogen atoms in the ester moiety versus comp. **35** with fluorine atoms in the NIH3T3 cells was two times higher (346 mg/kg versus 898 mg/kg, respectively).

(2) *Modification of Substituents at Position 4 of 3,4-Dihydro-2(1H)-pyridone Ring.* The series of 3,4-dihydro-2(1H)-pyridones with a phenyl substituent at position 4 of the pyridone ring and also the 4-unsubstituted ones were compared. In the case of 4-phenyl pyridone **34** and 4-unsubstituted pyridone **37**, both compounds containing the same N,N-dimethylcyclohexylammonium moiety and hydrogen atoms in the alkyl chain had no significant change of cytotoxicity on the tested cell lines. While in the case of 4-phenyl pyridone **35** and 4-unsubstituted pyridone **38**, both compounds containing the same N,N-

dimethylcyclohexylammonium moiety and fluorine atoms in the alkyl chain, the 4-unsubstituted **38** possessed a slightly higher cytotoxicity—LD₅₀ values were 553 mg/kg for comp. **38** and 898 mg/kg for comp. **35**.

(3) *Change of the Anion.* Change to perchlorate from the usual bromine anion (comp. **32** versus comp. **31**) did not give any influence on cytotoxicity on the tested cell lines.

(4) *Modification of the Cationic Moiety.* It is demonstrated that the dihydropyridone amphiphile **30** with a triphenylphosphonium polar head group was about 3 times less cytotoxic than the analogous dihydropyridone **31** with a pyridinium head group in the NIH3T3 cells, i.e., 1779 mg/kg versus 604 mg/kg. Change of pyridinium to N,N-dimethylcyclohexylammonium moiety as the cationic head group of dihydropyridone—comp. **31** versus comp. **35**—led to a slightly diminished cytotoxicity on cells in the case of comp. **35**. In this case, we observed the opposite relationship as for 1,4-DHP amphiphiles where it was demonstrated that quaternary ammonium surfactants are more toxic than their heterocyclic analogues. Toxicity of cationic lipids may be connected with the structure of their head groups [41].

The obtained data demonstrated that the 3,4-dihydro-2(1H)-pyridone amphiphiles (Table 2) with a pyridinium polar head group and introduced fluorine atoms in the ester moiety (comp. **31** and comp. **32**) were substantially more cytotoxic to tested cells than the structurally related 1,4-DHP amphiphiles **23** and **24**.

Most dihydropyridone series cationic amphiphiles show marked cytotoxicity towards cancer cells and medium cytotoxicity towards normal NIH3T3 cells: compounds possessing 1 or 2 long alkyl chains in ester and/or ammonium groups (with or without fluorine atoms on alkyl chains (comp. **31–34** and **36–38**)). There is an exclusion: comp. **30** possessing the triphenylphosphonium cationic group.

The obtained data allows one to choose compounds for putative use depending on their structure. Thus, due to the polyfluorinated alkyl ester groups in the DHP molecule, low toxicity (practically inert) amphiphilic compounds can be obtained (comp. **25**). Inert amphiphilic self-assembling compounds could be used as materials to form gene transfection or drug delivery nanoparticles for transmembrane transport according to the paradigm: pharmacologically inert transport vehicles should be used.

3.4.3. 2-Amino-3-alkoxycarbonylalkylammonium Halides (Table 3). The last group of delivery systems was formed by a 2-amino-3-alkoxycarbonylalkylammonium cationic moiety containing derivatives **39–48**.

(1) *Modification of the Cationic Moiety.* The cationic moiety was changed for 2-amino-3-hexadecyloxycarbonylammonium derivatives, namely, comp. **39–43**, and **45**. The obtained data demonstrated that N-(2-amino-4-(hexadecyloxy)-4-oxobut-2-en-1-yl)-N,N-dimethylcyclohexanaminium chloride (comp. **39**) shows the highest cytotoxicity in all the tested cell lines with a LD₅₀ value of 97 mg/kg, while alkyl moi-

ety containing compounds—N-(2-amino-4-(hexadecyloxy)-4-oxobut-2-en-1-yl)-N,N-dimethylhexan-1-aminium chloride (comp. **43**) and N-(2-amino-4-(hexadecyloxy)-4-oxobut-2-en-1-yl)-N,N-dimethyldodecan-1-aminium chloride (comp. **45**)—demonstrated lower cytotoxicity in all the tested cell lines with LD₅₀ values of 538 and 573 mg/kg, respectively.

(2) *Modification of the Ester Alkyl Groups.* The influence of the alkyl moiety was compared for two amphiphiles—comp. **48** with decyl ester versus comp. **45** with hexadecyl ester. The obtained LD₅₀ data show that the difference is not large—485 mg/kg versus 573 mg/kg, respectively.

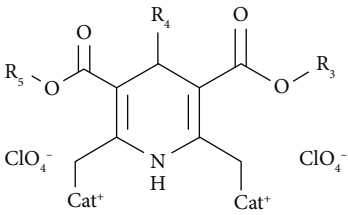
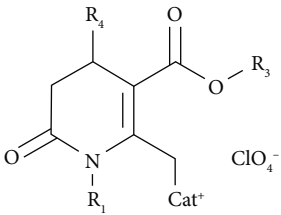
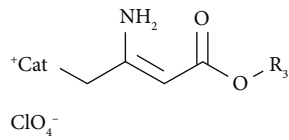
(3) *Change of the Anion.* The anions from the usual bromide comp. **44** were changed to chloride and also to iodide (comp. **45** and comp. **46**). According to IC₅₀ and LD₅₀ data, the change of the anion did not have any influence on the cytotoxicity in NIH3T3 cell lines; IC₅₀ values were around 30 µg/ml.

3.5. Electrochemical Oxidation. The electrochemical oxidation of various 1,4-dihydropyridine derivatives has been extensively studied [46–50] including 1,4-DHP derivatives containing cationic moieties [29, 44]. The electrochemical oxidation of the selected compounds studied in this work was performed by cyclic voltammetry on a stationary glassy carbon electrode in dry acetonitrile; the data is presented in Table 4. The perchlorates of the tested amphiphiles were obtained from the corresponding bromides by treating their abs. MeOH solutions with excess of conc. HClO₄ according to the procedure elaborated by Turovska et al. [44].

Now, we have used electrooxidation potentials to characterize electron donor properties of the studied compounds.

Compounds from the 1st group, containing cationic pyridinium methylene groups in positions 2 and 6 in the 1,4-DHP ring, have electrooxidation potentials of 1.57–1.58 V both in the case of 3,5-diethoxycarbonyl- and 3,5-didodecyloxycarbonyl-1,4-DHPs (comp. **1** and comp. **10**). This is also in agreement with our previous results, where the electrochemical oxidation potential of comp. **1** was determined as 1.7 V and electrochemical oxidation of this compound was demonstrated as a two-electron process [29]. Introduction of the CF₃ groups at the 4-carbon atom of a dodecyl chain in the ester moieties (comp. **18**) does not change the value of the electrooxidation potential, which is also 1.57 V. On the contrary, the addition of a CF₃ group in the pyridinium moiety in the 2 and 6 positions of the 1,4-DHP cycle (comp. **21**) leads to a slight increase of the electrooxidation potential (1.63 V). Moreover, a change of the 17 terminal H atoms to F atoms in one or both dodecyl chains leads to a further increase of the electrooxidation potential of 1.69–1.70 V (comp. **23** and comp. **24**). It should be noted that the parent compounds—1,4-DHP derivatives without cationic moieties—demonstrated lower electrooxidation potentials. Thus, 4-phenyl-substituted Hantzsch 1,4-dihydropyridine has a 1.08 V potential on a glassy carbon electrode [50] and the other 4-aryl-substituted 1,4-DHPs have 1.11 V potentials [51], but 4-monoalkyl-substituted 1,4-

TABLE 4: Oxidation potentials (E^{ox}) of selected compounds.

| <div style="display: flex; justify-content: space-around; align-items: flex-end;"> <div style="text-align: center;">  <p>1st group: comp. 1, 10, 21-24</p> </div> <div style="text-align: center;">  <p>2nd group: comp. 29, 38</p> </div> <div style="text-align: center;">  <p>3rd group: comp. 42, 44</p> </div> </div> | | | | | | | |
|---|-------------|-------------------|---|----------------|---|---|---------------------|
| Group | Comp. | R ¹ | R ³ | R ⁴ | R ⁵ | Cat ⁺ | E^{ox} (V) |
| 1st | 1 * | — | C ₂ H ₅ | Ph | C ₂ H ₅ | Py | 1.57 |
| | 10 * | — | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Py | 1.58 |
| | 21 * | — | C ₁₂ H ₂₅ | Ph | C ₁₂ H ₂₅ | Py-CF ₃ -4 | 1.63 |
| | 22 | — | C ₁₂ H ₂₄ CF ₃ | Ph | C ₁₂ H ₂₄ CF ₃ | Py | 1.57 |
| | 23 * | — | C ₁₂ H ₂₅ | Ph | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Py | 1.70 |
| | 24 * | — | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Ph | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Py | 1.69 |
| | 29 * | PhCH ₂ | CH ₃ | H | — | Py | 2.35 |
| 2nd | 38 * | H | (CH ₂) ₄ (CF ₂) ₇ CF ₃ | Ph | — | Py | 2.04 |
| 3rd | 42 * | — | C ₁₆ H ₃₃ | — | — | N-Me(CH ₂) ₂ N(CH ₂) ₂ Me | 1.49 |
| | 44 # | — | C ₁₆ H ₃₃ | — | — | N-Me ₂ C ₁₂ H ₂₅ | 1.24 |

*Original compounds—bromide; #original compound—chloride.

dihydropyridines at the same conditions have 1.01–1.03 V oxidation potentials [47].

Compounds from the 2nd group—amphiphilic 3,4-dihydropyridone derivatives with a pyridinium methylene moiety in position 6—have more positive electrooxidation potential. So, the unsubstituted at position 4 pyridone derivative **29** has an electrooxidation potential of 2.35 V. A compound possessing a phenyl substituent at position 4 and a 5-heptadecylfluorododecylcarboxy moiety in position 5 (comp. **38**) has a slightly lower oxidation potential (2.04 V), while the parent 4-unsubstituted or 4-phenyl-substituted 3,4-dihydropyridone derivatives without cationic moiety in position 6 have electrooxidation potentials of 1.52–1.64 V [52].

Compounds from the 3rd group—tested open-chain 2-amino-3-alkoxycarbonylalkylammonium halides **42** and **44**—have oxidation potentials of 1.49 V and 1.24 V, respectively.

Compounds from the 1st group (comp. **1–24**, Table 1) could be considered as analogues of 1,4-dihydronicotinamide and model compounds of redox coenzyme NAD(P)H. Many 4-aryl-1,4-DHPs possess antioxidant properties, including several Ca²⁺ channel blockers [53]. The antiradical activity (ARA) of two 1,4-DHPs containing cationic moieties was determined by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay; the results were expressed as a percentage (%) of the DPPH free radical scavenging, and the untreated level of the DPPH radical was designated as 100% [13, 31]. It was demonstrated that 3,5-didodecyloxycarbonyl-4-phenyl-1,4-dihydropyridine derivatives containing pyridinium moieties showed 25–60% radical scavenging activity which are comparable with the ARA of Diludin [54] (40%)—a widely

known antioxidant. Other 1,4-DHP amphiphiles containing saturated heterocyclic moieties—N-methylmorpholinium or N-methylpyrrolidinium derivatives—demonstrated more pronounced ARA, 95% and 54%, respectively. For 1,4-DHP amphiphiles possessing pyridinium moieties, the positive charge is delocalized in the heteroaromatic cycle, causing ARA reduction; for example, the electron donor dimethylamino group as a substituent of pyridinium moiety leads to a lower ARA (27%, comp. **13**) [13].

3.6. Determination of LogP and Molecular Topological Polar Surface Area. The lipophilicity of molecules represents their affinity for a lipophilic environment, and the lipophilicity may be expressed as logP [55, 56]. The molecular polar surface area (PSA) is a very useful parameter for the prediction of drug transport properties, and PSA is defined as a sum of the surfaces of polar atoms [57]. In practice, medicinal chemists use the PSA to quantify the polarity of drug molecules [58]. Data of the calculated topological polar surface area represent the compound's blood-brain barrier permeability [59, 60]. It allows one to plan further activities for the pleiotropic compounds.

Lipid-type compounds could be used as biologically active compounds *per se* or as transport vehicles or additives, so their lipophilicity (logP) and topological polar surface area (TPSA) were calculated, and the data are recorded in Table 5.

LogP values which surpass 5, according to Lipinski's Rule of Five, characterize compounds as lipophilic [61]. According to the obtained data for lipid-like amphiphiles, in some cases, the values of logP were <5, in particular for compounds **1**, **2**, **4**, **6**, **7**, **28**, and **29**. These compounds comprised short alkyl moieties in the ester groups. LogP values close to 5 were

TABLE 5: Log*P* and molecular topological polar surface area (TPSA) of selected compounds.

| Comp. | 1st group Log <i>P</i> | TPSA (Å ²) | Comp. | 2nd group Log <i>P</i> | TPSA (Å ²) | Comp. | 3rd group Log <i>P</i> | TPSA (Å ²) |
|-------|---------------------------|------------------------|-------|---------------------------|------------------------|-------|---------------------------|------------------------|
| 1 | 2.26 | 70.65 | 28 | 1.44 | 67.64 | 39 | 7.52 | 52.32 |
| 2 | 3.13 | 70.65 | 29 | 1.18 | 49.62 | 40 | 6.25 | 52.32 |
| 4 | 2.71 | 77.13 | 30 | 14.32 | 55.40 | 41 | 5.63 | 55.56 |
| 6 | 3.18 | 70.65 | 31 | 9.43 | 58.41 | 42 | 5.44 | 55.56 |
| 7 | 3.20 | 98.34 | 32 | 9.43 | 58.41 | 43 | 7.98 | 52.32 |
| 10 | 10.97 | 70.65 | 33 | 6.19 | 55.40 | 44 | 10.65 | 52.32 |
| 12 | 11.63 | 70.65 | 34 | 10.01 | 55.40 | 47 | 5.40 | 55.33 |
| 13 | 11.42 | 77.13 | 35 | 14.81 | 55.40 | 48 | 7.98 | 52.32 |
| 17 | 13.55 | 64.63 | 36 | 4.70 | 55.40 | | | |
| 20 | 19.81 | 64.63 | 37 | 8.52 | 55.40 | | | |
| 21 | 12.81 | 70.65 | 38 | 13.32 | 55.40 | | | |
| 22 | 13.22 | 70.65 | | | | | | |
| 23 | 15.77 | 70.65 | | | | | | |
| 24 | 18.64 | 70.65 | | | | | | |
| 25 | 24.68 | 70.65 | | | | | | |

obtained for compounds **36**, **41**, **42**, and **47**, namely, 4.70, 5.63, 5.44, and 5.40, respectively. Log*P* values for dicationic 1,4-DHP amphiphiles possessing longer alkyl chains or fluorinated alkyl groups in the ester moieties were determined in the 11–24.5 interval, while for long alkyl ester moieties containing 3,4-dihydropyridone amphiphiles, log*P* values were determined in the 6–15 interval. The difference could be due to the number of alkyl groups. Log*P* values for other open-chain compounds were determined in the 6–11 interval.

Nevertheless, TPSA never surpasses 90, so the compounds are prone to permeate cells; additionally, they can penetrate the blood-brain barrier [62]. Among all tested amphiphiles, only for 1,1'-((4-(2-(difluoromethoxy)phenyl)-3,5-bis((propoxymethoxy)carbonyl)-1,4-dihydropyridine-2,6-diyl)bis(methylene))bis(pyridin-1-ium) dibromide (comp. **7**) was the TPSA value higher than 90, i.e., 98.34 Å². This could be explained by the influence of the structure components of the compound. TPSA values for the other compounds were defined in accordance with the main fragments of the compound structures. So, for the other 1st group compounds—dicationic 1,4-DHP amphiphiles—TPSA values were in the 71–77 Å² interval; for the 2nd group compounds—cationic 3,4-dihydropyridone amphiphiles—TPSA values were in the 50–58 Å² interval with the exception of compound **28** which had a TPSA value of 67.64 Å²; and for the 3rd group compounds—open-chain 2-amino-3-alkoxycarbonylalkylammonium cationic moiety containing amphiphiles—the TPSA values were in the 52–56 Å² interval.

4. Conclusions

Polyfunctional self-assembling synthetic lipid-like compounds, such as pharmacological and chemotherapeutical agents, namely, 3,5-dialkoxycarbonyl-1,4-dihydropyridines

(1,4-DHPs) comprising pyridinium or ammonium substituents at the 2 and 6 positions; structurally related compounds, derivatives of 3,4-dihydro-2-oxopyridines as isomeric 3,4-dihydropyridine structures with an additional intracyclic carbonyl group; and the N-β-carbonylvinylamido system, namely, 2-amino-3-alkoxycarbonylalkylammonium halides as open chain analogues of the first type of the abovementioned compounds, were studied. The main properties and major functions of these compounds are their amphiphilic character, liposome-forming ability, RNA transfection (by self-assembling compounds), antiradical and antioxidant properties, growth regulation—both in malignant and non-malignant cell types—anticancer properties due to cytotoxicity, and MDR inhibition [13, 16, 27, 28, 31, 33].

In this work we have demonstrated biological properties of cationic 1,4-dihydropyridine as self-assembling synthetic lipids and dihydropyridones as well as open-chain analogues: their cytotoxicity against cancer cell lines HT-1080 and MH-22A in comparison with cytotoxicity against normal NIH3T3 cells. The obtained data showed that 1,4-DHP derivatives containing cationic moieties in positions 2 and 6 and possessing dodecyl alkyl chains in the ester groups in positions 3 and 5 demonstrated high cytotoxicity on cancer cells HT-1080 and MH-22A, but low cytotoxicity on noncancerous NIH3T3 cells. According to our previous studies, these compounds also demonstrated significant antiradical activity and also gene delivery activity [13], and for some of them, reversal of multidrug resistance in murine lymphoma cells [16]. Together with antiradical activity, cell growth regulation, multidrug resistance inhibition, nucleic acid delivery, and the polyfunctional (pleiotropic) type of properties of the mentioned compounds open new avenues for their studies and use. According to literature data, liposomes could be used not only to transport biologically active compounds but also to have their own specific biological activity, e.g., to protect cells and encapsulated components against oxidative

damage. Liposomes are proposed for the delivery of antioxidants for protection against pathological conditions related to oxidative stress [63]. In our case, liposomes could be used *per se* to protect against oxidative damage.

A calculated degree of lipophilicity and TPSA data can be used to choose compounds according to their permeability through membranes, including the blood-brain barrier, to guide them to the proper location. It was demonstrated that membrane permeability in a variety of systems, including model liposome bilayers, various cells, and epidermal tissue, correlated strongly with data regarding hydrocarbon-water partition coefficients [64]. TPSA values for selected compounds were defined in accordance with the main fragments of compound structures. The determined $\log P$ values were highest for dodecyl ester groups in positions 3 and 5 of the 1,4-DHP and lowest for short alkyl chain containing amphiphiles.

This study also revealed the correlation of the cytotoxic effects of 3 groups of structurally related synthetic cationic lipids according to their molecular structures. The results indicated that among the tested compound groups, amphiphiles based on the 1,4-DHP core demonstrated high cytotoxicity in cancer cells HT-1080 and MH-22A, but low cytotoxicity in the noncancerous NIH3T3 cells.

The obtained results may serve as guidelines for the development of drug formulations to be used in cancer treatment on the basis of these pleiotropic lipid-like 1,4-DHP amphiphiles.

Data Availability

The experimental data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This research was supported by the PostDocLatvia Project Nr.1.1.1.2/VIAA/2/18/371 “Bifunctional Amphiphilic Lipid-Like Compounds—Self-Assembling Properties and Biological Activities” (to M. Rucins) and EuroNanoMed2 Project INNOCENT “Innovative Nanopharmaceuticals: Targeting Breast Cancer Stem Cells by a Novel Combination of Epigenetic and Anticancer Drugs with Gene Therapy.”

Supplementary Materials

Scheme S1: synthesis of 1,4-dihydropyridine (1,4-DHP) amphiphiles **1–7**, **9–22**, and **26**. Scheme S2: synthesis of unsymmetrical 1,4-DHP amphiphile **23**. Scheme S3: synthesis of 3,4-dihydro-2(1H)-pyridone (DHPDO) amphiphiles **28–38**. Scheme S4: synthesis of 2-amino-3-alkoxycarbonylalkylammonium halides **39–48**. Physicochemical characterization of new compounds is provided. (*Supplementary Materials*)

References

- [1] T. Olusanya, R. H. Ahmad, D. Ibegbu, J. Smith, and A. Elkordy, “Liposomal drug delivery systems and anticancer drugs,” *Molecules*, vol. 23, no. 4, p. 907, 2018.
- [2] C. T. Inglut, A. J. Sorrin, T. Kuruppu et al., “Immunological and toxicological considerations for the design of liposomes,” *Nanomaterials*, vol. 10, no. 2, p. 190, 2020.
- [3] A. Carmona-Ribeiro, “Bilayer-forming synthetic lipids: drugs or carriers?,” *Current Medicinal Chemistry*, vol. 10, no. 22, pp. 2425–2446, 2003.
- [4] A. Lechanteur, V. Sanna, A. Duchemin, B. Evrard, D. Mottet, and G. Piel, “Cationic liposomes carrying siRNA: impact of lipid composition on physicochemical properties, cytotoxicity and endosomal escape,” *Nanomaterials*, vol. 8, no. 5, p. 270, 2018.
- [5] K. He and M. Tang, “Safety of novel liposomal drugs for cancer treatment: advances and prospects,” *Chemico-Biological Interactions*, vol. 295, pp. 13–19, 2018.
- [6] L. Milkovic, W. Siems, R. Siems, and N. Zarkovic, “Oxidative stress and antioxidants in carcinogenesis and integrative therapy of cancer,” *Current Pharmaceutical Design*, vol. 20, no. 42, pp. 6529–6542, 2014.
- [7] Q. Cui, J.-Q. Wang, Y. G. Assaraf et al., “Modulating ROS to overcome multidrug resistance in cancer,” *Drug Resistance Updates*, vol. 41, pp. 1–25, 2018.
- [8] H. Sies and D. P. Jones, “Reactive oxygen species (ROS) as pleiotropic physiological signalling agents,” *Nature Reviews. Molecular Cell Biology*, vol. 21, no. 7, pp. 363–383, 2020.
- [9] H. Sies, “Oxidative stress: a concept in redox biology and medicine,” *Redox Biology*, vol. 4, pp. 180–183, 2015.
- [10] A. Garu, G. Moku, S. K. Gulla et al., “Examples of tumor growth inhibition properties of liposomal formulations of pH-sensitive histidinylated cationic amphiphiles,” *ACS Biomaterials Science & Engineering*, vol. 1, no. 8, pp. 646–655, 2015.
- [11] Z. Fasili, F. Mehri, H. A. Ebrahimi et al., “Applying nanoparticles in the treatment of viral infections and toxicological considerations,” *Pharmaceutical and Biomedical Research*, vol. 5, no. 4, pp. 1–20, 2019, <http://pbr.mazums.ac.ir/article-1-247-en.html>.
- [12] J. Barar and Y. Omid, “Intrinsic bio-signature of gene delivery nanocarriers may impair gene therapy goals,” *BioImpacts: BI*, vol. 3, no. 3, pp. 105–109, 2013.
- [13] K. Pajuste, Z. Hyvonen, O. Petrichenko et al., “Gene delivery agents possessing antiradical activity: self-assembling cationic amphiphilic 1,4-dihydropyridine derivatives,” *New Journal of Chemistry*, vol. 37, no. 10, pp. 3062–3075, 2013.
- [14] O. Petrichenko, M. Rucins, A. Vezane et al., “Studies of the physicochemical and structural properties of self-assembling cationic pyridine derivatives as gene delivery agents,” *Chemistry and Physics of Lipids*, vol. 191, pp. 25–37, 2015.
- [15] G. Apsite, I. Timofejeva, A. Vezane et al., “Synthesis and comparative evaluation of novel cationic amphiphile C12-Man-Q as an efficient DNA delivery agent in vitro,” *Molecules*, vol. 23, no. 7, p. 1540, 2018.
- [16] M. Cindric, A. Cipak, J. Serly et al., “Reversal of multidrug resistance in murine lymphoma cells by amphiphilic dihydropyridine antioxidant derivative,” *Anticancer Research*, vol. 30, no. 10, pp. 4063–4069, 2010.

- [17] G. Dubur, B. Vígante, A. Plotniece et al., "Dihydropyridine derivatives as bioprotectors," *Chimica Oggi*, vol. 26, no. 2, pp. 68–70, 2008.
- [18] M. Rucins, P. Dimitrijevs, K. Pajuste et al., "Contribution of molecular structure to self-assembling and biological properties of bifunctional lipid-like 4-(N-alkylpyridinium)-1,4-dihydropyridines," *Pharmaceutics*, vol. 11, no. 3, p. 115, 2019.
- [19] M. Rucins, D. Kaldre, K. Pajuste et al., "Synthesis and studies of calcium channel blocking and antioxidant activities of novel 4-pyridinium and/or N-propargyl substituted 1,4-dihydropyridine derivatives," *Comptes Rendus Chimie*, vol. 17, no. 1, pp. 69–80, 2014.
- [20] M. Petrova, R. Muhamadejev, B. Vígante et al., "Intramolecular C-H...O hydrogen bonding in 1,4-dihydropyridine derivatives," *Molecules*, vol. 16, no. 9, pp. 8041–8052, 2011.
- [21] K. Saotome, H. Morita, and M. Umeda, "Cytotoxicity test with simplified crystal violet staining method using microtitre plates and its application to injection drugs," *Toxicology in Vitro*, vol. 3, no. 4, pp. 317–321, 1989.
- [22] W. S. Stokes, S. Casati, J. Strickland, and M. Paris, "Neutral red uptake cytotoxicity tests for estimating starting doses for acute oral toxicity tests," *Current Protocols in Toxicology*, vol. 36, no. 1, 2008.
- [23] Interagency Coordinating Committee on the Validation of Alternative Methods, ICCVAM test method evaluation report (TMER): in vitro cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity testing, 2006, NIH Publication No: 07-4519.
- [24] The national toxicology program (NTP) Interagency center for the evaluation of alternative toxicological methods (NICEATM), Background review document: in vitro cytotoxicity test methods for estimating acute oral systemic toxicity, vol. 1, 2006 June 2020 https://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/brd_tmter/brdvol1_nov2006.pdf.
- [25] The national toxicology program (NTP) Interagency center for the evaluation of alternative toxicological methods (NICEATM), Background review document: in vitro cytotoxicity test methods for estimating acute oral systemic toxicity, vol. 2, 2006 June 2020 https://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/brd_tmter/brdvol2_nov2006.pdf.
- [26] EP, Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC), Publications office of the European Union, 2008, doi:2004R0726 - v.7 of 05.06.2013.
- [27] G. Apsīte, E. Bisenieks, R. Brūvere et al., Novel biologically active enaminoester derivatives as agents for chemotherapy, LV15347B, Espacenet, 2019.
- [28] Z. Hyvönen, A. Plotniece, I. Reine, B. Chekavichus, G. Duburs, and A. Urtti, "Novel cationic amphiphilic 1,4-dihydropyridine derivatives for DNA delivery," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1509, no. 1-2, pp. 451–466, 2000.
- [29] A. Plotniece, K. Pajuste, D. Kaldre et al., "Oxidation of cationic 1,4-dihydropyridine derivatives as model compounds for putative gene delivery agents," *Tetrahedron*, vol. 65, no. 40, pp. 8344–8349, 2009.
- [30] K. Pajuste, I. Krivicka, M. Plotniece et al., The synthesis of new cationic 1,4-dihydropyridine derivatives for DNA delivery, Rigas Teh. Univ. Zinat. Raksti, Ser. 1 Materialzinat. Un Lie-tiska Kim, vol. 11, pp. 7–10, 2005, July 2020 <https://ortus.rtu.lv/science/lv/publications/6433>.
- [31] I. Bruvere, E. Bisenieks, J. Poikans et al., "Dihydropyridine derivatives as cell growth modulators in vitro," *Oxidative Medicine and Cellular Longevity*, vol. 2017, 15 pages, 2017.
- [32] R. Muhamadejev, M. Petrova, R. Smits et al., "Study of interactions of mononucleotides with 1,4-dihydropyridine vesicles using NMR and ITC techniques," *New Journal of Chemistry*, vol. 42, no. 9, pp. 6942–6948, 2018.
- [33] R. Smits, Y. Goncharenko, I. Vesere et al., "Synthesis and self-assembly of novel fluorous cationic amphiphiles with a 3,4-dihydro-2(1H)-pyridone spacer," *Journal of Fluorine Chemistry*, vol. 132, no. 6, pp. 414–419, 2011.
- [34] M. Rucins, K. Pajuste, A. Sobolev et al., "Data for the synthesis and characterisation of 2,6-di(bromomethyl)-3,5-bis(alkoxycarbonyl)-4-aryl-1,4-dihydropyridines as important intermediates for synthesis of amphiphilic 1,4-dihydropyridines," *Data in Brief*, vol. 30, p. 105532, 2020.
- [35] K. Pajuste, A. Plotniece, K. Kore et al., "Use of pyridinium ionic liquids as catalysts for the synthesis of 3,5-bis(dodecyloxycarbonyl)-1,4-dihydropyridine derivative," *Central European Journal of Chemistry*, vol. 9, no. 1, pp. 143–148, 2011.
- [36] P. G. Nantermet, J. C. Barrow, H. G. Selnick et al., "Selective α 1a adrenergic receptor antagonists based on 4-aryl-3,4-dihydropyridine-2-ones," *Bioorganic & Medicinal Chemistry Letters*, vol. 10, no. 15, pp. 1625–1628, 2000.
- [37] K. B. Goodman, H. Cui, S. E. Dowdell et al., "Development of dihydropyridone indazole amides as selective rho-kinase inhibitors," *Journal of Medicinal Chemistry*, vol. 50, no. 1, pp. 6–9, 2007.
- [38] X. Huang, S. Broadbent, C. Dvorak, and S. H. Zhao, "Pilot-plant preparation of 3,4-dihydropyridin-2-one derivatives, the core structures of P2X7 receptor antagonists," *Organic Process Research and Development*, vol. 14, no. 3, pp. 612–616, 2010.
- [39] K. T. Homan, K. M. Larimore, J. M. Elkins, M. Szklarz, S. Knapp, and J. J. G. Tesmer, "Identification and structure-function analysis of subfamily selective g protein-coupled receptor kinase inhibitors," *ACS Chemical Biology*, vol. 10, no. 1, pp. 310–319, 2014.
- [40] A. Morales, E. Ochoa, M. Suárez et al., "Novel hexahydrofuro[3,4-b]-2(1H)-pyridones from 4-aryl substituted 5-alkoxycarbonyl-6-methyl-3,4-dihydropyridones," *J. Heterocycl. Chem.*, vol. 33, no. 1, pp. 103–107, 1996.
- [41] B. Vígante, A. Plotniece, M. Rucins et al., "An efficient synthesis of multisubstituted 4-nitrobuta-1,3-dien-1-amines and application in cyclisation reactions," *Tetrahedron*, vol. 74, no. 21, pp. 2596–2607, 2018.
- [42] B. Stanovnik, "Enaminone, enaminoesters, and related compounds in the metal-free synthesis of pyridines and fused pyridines," *European J. Org. Chem.*, vol. 2019, no. 31-32, pp. 5120–5132, 2019.
- [43] J. Wang, X. Guo, Y. Xu, L. Barron, and F. C. Szoka, "Synthesis and characterization of long chain alkyl acyl carnitine esters. Potentially biodegradable cationic lipids for use in gene delivery," *Journal of Medicinal Chemistry*, vol. 41, no. 13, pp. 2207–2215, 1998.
- [44] B. Turovska, J. Stradins, I. Turovskis, A. Plotniece, A. Shmidlers, and G. Duburs, "Electrochemical oxidation of compounds containing 1,4-dihydropyridine and pyridinium rings - analogs of gene transfection agents," *Chemistry of Heterocyclic Compounds*, vol. 40, no. 6, pp. 753–758, 2004.

- [45] H. Lv, S. Zhang, B. Wang, S. Cui, and J. Yan, "Toxicity of cationic lipids and cationic polymers in gene delivery," *Journal of Controlled Release*, vol. 114, no. 1, pp. 100–109, 2006.
- [46] L. Baumane, A. Krauze, S. Belyakov et al., "Synthesis, structure, and electrochemical characteristics of 4-aryl-2-carbamoylmethylthio-5-ethoxycarbonyl-1,4-dihydropyridine-3-carboxylic acid nitriles," *Chemistry of Heterocyclic Compounds*, vol. 41, no. 3, pp. 362–373, 2005.
- [47] B. Turovska, I. Goba, I. Turovskis et al., "Electrochemical oxidation of 4-monoalkyl-substituted 1,4-dihydropyridines," *Chemistry of Heterocyclic Compounds*, vol. 44, no. 12, pp. 1483–1490, 2008.
- [48] L. J. Núñez-Vergara, C. López-Alarcón, P. A. Navarrete-Encina, A. M. Atria, C. Camargo, and J. A. Squella, "Electrochemical and EPR characterization of 1,4-dihydropyridines. Reactivity towards alkyl radicals," *Free Radical Research*, vol. 37, no. 1, pp. 109–120, 2009.
- [49] L. J. Núñez-Vergara, R. Salazar, C. Camargo et al., "Oxidation of 4-hydroxyphenyl 1,4-dihydropyridines in dimethylsulfoxide and its reactivity towards alkylperoxyl radicals in aqueous medium," *Bioorganic & Medicinal Chemistry*, vol. 15, no. 12, pp. 4318–4326, 2007.
- [50] V. Pardo-Jiménez, C. Barrientos, K. Pérez-Cruz et al., "Synthesis and electrochemical oxidation of hybrid compounds: dihydropyridine-fused coumarins," *Electrochimica Acta*, vol. 125, pp. 457–464, 2014.
- [51] Y. P. Stradyn, Y. I. Beilis, Y. R. Uldrikis, G. Y. Dubur, A. E. Sausin, and B. S. Chekavichus, "Voltamperometry of 1,4-dihydropyridine derivatives," *Chemistry of Heterocyclic Compounds*, vol. 11, no. 11, pp. 1299–1303, 1975.
- [52] R. Smits, B. Turovska, S. Belyakov, A. Plotniece, and G. Duburs, "Synthesis of 5-carboxy-6-methyl-3,4-dihydro-2(1H)-pyridone derivatives and their electrochemical oxidation to 2-pyridones," *Chemical Physics Letters*, vol. 649, pp. 84–87, 2016.
- [53] A. Augustyniak, G. Bartosz, A. Čipak et al., "Natural and synthetic antioxidants: an updated overview," *Free Radical Research*, vol. 44, no. 10, pp. 1216–1262, 2010.
- [54] G. Tirzitis, D. Tirzite, and Z. Hyvonen, "Antioxidant activity of 2,6-dimethyl-3,5-dialkoxycarbonyl-1,4-dihydropyridines in metal-ion catalyzed lipid peroxidation," *Czech Journal of Food Sciences*, vol. 19, no. 3, pp. 81–84, 2013.
- [55] S. A. Wildman and G. M. Crippen, "Prediction of physicochemical parameters by atomic contributions," *Journal of Chemical Information and Computer Sciences*, vol. 39, no. 5, pp. 868–873, 1999.
- [56] G. H. Goetz and M. Shalaeva, "Leveraging chromatography based physicochemical properties for efficient drug design," *ADMET DMPK*, vol. 6, no. 2, pp. 85–104, 2018.
- [57] P. Ertl, B. Rohde, and P. Selzer, "Fast calculation of molecular polar surface area as a sum of fragment-based contributions and its application to the prediction of drug transport properties," *Journal of Medicinal Chemistry*, vol. 43, no. 20, pp. 3714–3717, 2000.
- [58] G. Caron and G. Ermondi, "Molecular descriptors for polarity: the need for going beyond polar surface area," *Future Medicinal Chemistry*, vol. 8, no. 17, pp. 2013–2016, 2016.
- [59] Z. Wang, H. Yang, Z. Wu et al., "In silico prediction of blood-brain barrier permeability of compounds by machine learning and resampling methods," *ChemMedChem*, vol. 13, no. 20, pp. 2189–2201, 2018.
- [60] S. Benfeito, C. Oliveira, C. Fernandes et al., "Fine-tuning the neuroprotective and blood-brain barrier permeability profile of multi-target agents designed to prevent progressive mitochondrial dysfunction," *European Journal of Medicinal Chemistry*, vol. 167, pp. 525–545, 2019.
- [61] C. A. Lipinski, "Drug-like properties and the causes of poor solubility and poor permeability," *Journal of Pharmacological and Toxicological Methods*, vol. 44, no. 1, pp. 235–249, 2000.
- [62] S. A. Hitchcock and L. D. Pennington, "Structure-brain exposure relationships," *Journal of Medicinal Chemistry*, vol. 49, no. 26, pp. 7559–7583, 2006.
- [63] Z. E. Suntres, "Liposomal antioxidants for protection against oxidant-induced damage," *Journal of Toxicology*, vol. 2011, Article ID 152474, 16 pages, 2011.
- [64] M. R. Naylor, A. M. Ly, M. J. Handford et al., "Lipophilic permeability efficiency reconciles the opposing roles of lipophilicity in membrane permeability and aqueous solubility," *Journal of Medicinal Chemistry*, vol. 61, no. 24, pp. 11169–11182, 2018.