Article

Synthesis and Critical View on the Structure-Activity Relationships of *N*-(Substituted phenyl)-/*N*-Diphenylmethyl-piperazine-Based Conjugates as Antimycobacterial Agents

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Abstract: This research focused on a three-step synthesis, analytical, physicochemical, and biological evaluation of hybrid molecules **6a–g**, containing a lipophilic 3-trifluoromethylphenyl moiety, polar carbamoyloxy bridge, 2-hydroxypropan-1,3-diyl chain and 4-(substituted phenyl)-/4-diphenylmethylpiperazin-1-ium-1-yl fragment. The estimation of analytical and physicochemical descriptors (m/z_{measured} via HPLC-UV/HR-MS, $\log \epsilon_2(\text{Ch-T})$ from UV/Vis spectrophotometry and $\log k_w$ via RP-HPLC) as well as in vitro antimycobacterial and cytotoxic screening of given compounds were carried out (i.e., determination of MIC and IC_{50} values). These highly lipophilic molecules ($\log k_w = 4.1170\text{--}5.2184$) were tested against *Mycobacterium tuberculosis* H₃₇R_a ATCC 25177 (*Mtb* H₃₇R_a), *M. kansasii* DSM 44162 (MK), *M. smegmatis* ATCC 700084 (MS), and *M. marinum* CAMP 5644 (MM). The impact of the **6a–g** set on the viability of human liver hepatocellular carcinoma (HepG2) cells was also investigated. 1-[2-Hydroxypropyl]-{(3-trifluoromethyl)-phenyl}carbamoyloxy]-4-(3,4-dichlorophenyl)piperazin-1-ium chloride (**6e**) and 1-[2-hydroxy-propyl]-{(3-trifluoromethyl)phenyl}carbamoyloxy]-4-(4-diphenylmethyl)piperazin-1-ium chloride (**6g**) most effectively inhibited the growth of *Mtb* H₃₇R_a (MIC < 3.80 μM). The substance **6g** also showed interesting activity against MM (MIC = 8.09 μM). All obtained data served as input values for structure-activity relationship evaluations using statistical principal component analysis. In fact, the toxicity of both **6e** ($\text{IC}_{50} = 29.39 \mu\text{M}$) and **6g** ($\text{IC}_{50} = 22.18 \mu\text{M}$) in HepG2 cells as well as selectivity index (SI) values (SI < 10.00) prevented to consider these promising antimycobacterials safe.

Keywords: drug development; *N*-arylpiperazines; mycobacteria; electronic properties; lipophilicity; cytotoxicity; structure-activity

1. Introduction

N-containing heterocycles are exceptionally valuable structures that hold unique positions in the design and development of biologically active compounds. *N*-Arylpiperazines have been considered privileged scaffolds because they interact with multiple pharmacological targets with (much) higher affinity and selectivity than other fragments [1–5].

In addition, biologically effective molecules containing these privileged scaffolds usually provide convenient drug-like properties [6]. The *N*-arylpiperazine ligands can also bind to specific effector structures of mycobacteria affecting various biochemical processes within these pathogens. Overall, given scaffolds might serve as a very suitable structural platform to develop compounds with multiple pharmacological activities, including the antimycobacterial ones, or they might be involved in the construction of compound libraries [7,8]. The search for effective antimycobacterials with favorable pharmacokinetic and toxicological properties is still challenging, because infections caused by tuberculous and non-tuberculous mycobacterial strains are responsible for significant morbidity and mortality worldwide. Moreover, current antibiotic treatment options often result in suboptimal outcomes [9].

Girase et al. (2021) reported that quite a considerable number of the compounds containing an *N*-arylpiperazine structural motif showed remarkable activity against *Mycobacterium tuberculosis* H₃₇R_v (*Mtb* H₃₇R_v), H₃₇R_a (*Mtb* H₃₇R_a), *M. smegmatis* (MS), and other mycobacterial strains. The authors also provided structure-activity relationships (SARs) for particular classes of these molecules divided according to their chemical structure. However, there is still room for the development of novel, fast-acting, potent, and safe antimycobacterial agents [10].

The design of antimycobacterial drugs via conjugation of efficacious drug fragments is well-known. Resulting compounds might be able to simultaneously affect different biological targets, exerting multiple biological actions, or one part of this molecule can offset adverse effects caused by the other one [11–14]. Upadhayaya et al. (2010) structurally combined a highly lipophilic 2-naphthyl(phenyl)methyl moiety (A) with a polar etheric bridge (B), 2-hydroxypropan-1,3-diyl chain (C) and *N*-aryl-/*N*-(substituted)phenyl-/*N*-benzyl-/*N*-diphenylmethylpiperazine group (D) [15] providing a series of RU compounds (Figure 1).

Figure 1. General structure of naphthalene-based RU derivatives investigated in vitro as well as in silico against *Mycobacterium tuberculosis* H₃₇R_v. (* = chiral center).

The authors preliminary explored SARs and concluded that electron-withdrawing (EW) lipophilic substituents (i.e., *R* substituent or disubstitution), attached to a phenyl ring of these molecules, decreased in vitro efficiency notably or even caused its loss against *Mtb* H₃₇R_v. Paradoxically, bulky lipophilic moieties at a piperazin-1,4-diyl heterocycle improved the activity. The most effective compounds containing a benzyl fragment (*W* = benzyl) or diphenylmethyl group (*W* = diphenylmethyl) inhibited the growth of *Mtb* H₃₇R_v (the inhibition of 91% and 98%, respectively) showing the minimum inhibitory concentration (MIC) value of 13.41 and 11.53 μM , respectively. The decrease in electron density on ring C atoms due to the replacement of phenyl with 2-pyridyl (Figure 1) caused the loss of the ability to inhibit the growth of given slow-growing pathogens [15].

The proper selection of these substituents is essential for interactions between resulting derivatives and particular amino acids localized at a binding site of adenosine-5'-triphosphate (ATP) synthase [15]. The enzyme is strongly evolutionarily conserved among prokaryotes and eukaryotes. Oxidative phosphorylation, as a crucial process for the existence of *Mtb* H₃₇R_v obligate aerobic pathogens, leads to the ATP production necessary for growth and survival. Apart from replicating mycobacteria, ATP synthase is also

essential in the dormant state, because it carries specific features facilitating survival under nonreplicating conditions [16,17]. When replacing a benzyl group ($W = \text{benzyl}$) with the bulkier diphenylmethyl ($W = \text{dimethylphenyl}$) one, an extended geometry is provided at the binding site. This modification increases the compound's interactions with this site, as a docking study predicted, thus improving in vitro anti-*Mtb* activity [15].

Other researchers suggested very precise modifications of fragment **A**, which can be made without the loss of in vitro antimycobacterial efficacy. For example, its replacement with a 3-/4-alkoxyphenyl group (alkoxy = methoxy to butoxy) was acceptable assuming simultaneous convenient structural changes within both **B** and **D** compartments. If such structural arrangement is established, the introduction of lipophilic substituents (3'-CF₃ or 4'-F) at a 4-(substituted)phenylpiperazin-1-ium-1-yl moiety improved in vitro activity against *Mtb* H₃₇R_v, *M. kansasii* DSM 44162 (MK DSM), and several other mycobacteria as well [18,19]. In addition, the positive impact of increased lipophilicity on anti-tuberculosis (anti-TB) activity was also supported with the conclusions published in [20–22].

The replacement of a piperazin-1,4-diyl heterocycle with a piperidin-1,4-diyl moiety together with the introduction of a 4-OH group retained high in vitro anti-TB efficiency of the resulting compound **SD** (Figure 2) if both 4'-Cl-3'-CF₃-phenyl and 4-Cl-phenyl groups were present. Interestingly, the replacement of the latter with phenyl, 3-CF₃-phenyl, or a 3,4-diCl-phenyl fragment decreased anti-TB activity [23].

Figure 2. The highly lipophilic compound **SD** was efficient in vitro against *Mycobacterium tuberculosis* H₃₇R_v. (* = chiral center).

The OH group within compartment **C** (Figure 1) is important for the activity of the series of **RU** compounds against *Mtb* H₃₇R_v [15]. Docking studies indicated hydrogen bonding interaction(s) between a given moiety and highly conserved and charged residues of particular amino acids localized at the pathogen's ATP synthase binding site. The synthase is an enzyme consisting of two functional domains, i.e., ATP-synthesizing F₁ unit and proton-translocating F₀ unit [24]. The research in [25] also confirmed that such structural arrangement of molecules, i.e., the combination of a lipophilic group, polar fragment (O-bridge), 2-hydroxypropan-1,3-diyl chain and 4-aryl piperazin-1-yl moiety, was favorable in order to interact with the synthase.

The linker might also be modified precisely (Figure 3) to afford effective molecules from a series of **VN** compounds against multidrug-resistant TB (MDR-TB). The modification provided different mechanisms of anti-MDR-TB action, as the designed derivatives selectively targeted and inhibited DNA-dependent RNA polymerase enzymes of the pathogens. The keto-enol functional moiety (Figure 3) is responsible for chelating Mg²⁺ ions located in the β'-subunit of a given enzyme [26].

Figure 3. General structure of highly lipophilic anti-MDR-TB compounds (series VN) containing a keto-enol functional group as a part of their connecting chain.

The current research aimed at the synthesis of highly lipophilic racemates (Figure 4) structurally similar to those characterized in the literature [15,18,19] and estimation of the parameters describing their spectral, electronic, and lipohydrophilic properties. The structural framework of designed **6a–g** allowed for the assumption of their noticeable in vitro activity against some of the tested mycobacteria, i.e., an attenuated *Mtb* H₃₇R_a ATCC 25177, *MK* DSM, *M. smegmatis* ATCC 700084 (*MS*), and *M. marinum* CAMP 5644 (*MM*).

Figure 4. Synthesis of the intermediates (i.e., **3** and **5a–g**) and in vitro biologically screened compounds (i.e., **6a–g**). Reagents and conditions: (i) anhydrous toluene, continuous stirring at 70 °C for 25 h; (ii) anhydrous propan-2-ol, continuous stirring at reflux for 20 h; (iii) saturated solution of hydrogen chloride in diethyl ether. (* = chiral center).

The impact of **6a–g** on the viability of human liver hepatocellular carcinoma (HepG2) cells was also investigated in vitro. The cell line shows high proliferation rates and epithelial-like morphology [27], thus being used most commonly in drug metabolism and hepatotoxicity studies.

Despite the fact that a stereogenic center can be found in the structure of proposed compounds **6a–g**, the synthesis of racemic molecules has been a rational and convenient strategy, especially in the early phases of conceptual development of (not only) antimycobacterial drugs to confirm the relevance of the chosen structural motifs [15,18,19,28]. The replacement of an O-bridge (**B**; Figure 1) with an NHCOO moiety might be advantageous.

The derivatives **6a–g** (Figure 4) would not passively cross the blood–brain barrier because of the increase in number of polar O and N atoms [29].

2. Materials and Methods

2.1. Chemistry

2.1.1. General Information

All information related to reagents used for syntheses, drying of solvents, and thin-layer chromatography (TLC) for reactions monitoring and preliminary purity determination for all synthesized or analyzed compounds (i.e., **3**, **5a–g**, and **6a–g**) can be found in [30]. The details regarding yields, determinations of melting point (Mp) values, and estimations of R_f parameters (TLC) for those molecules (Figure 1) are already published [30] and are also available in the Supplementary Materials. General procedures for the preparation of the molecules (**3**, **5a–g**, and **6a–g**) can be found in a research paper by Pospisilova et al. (2020) [30].

The equipment, employed software tools, and particular methods to measure and interpret properly infrared (IR) spectra, nuclear magnetic resonance (NMR) spectra (^1H NMR, ^{13}C NMR, and ^{19}F NMR), high-performance liquid chromatography high-resolution mass spectra (HPLC-UV/HR-MS) as well as ultraviolet/visible (UV/Vis) spectra for all synthesized or analyzed molecules (i.e., **3**, **5a–g**, and **6a–g**) can be found in [30]; in addition, they are also provided in the Supplementary Materials.

The m/z values of observed $[\text{M}+\text{H}]^+$ adducts for all investigated molecules were obtained (m/z_{measured}) from current HPLC-UV/HR-MS analyses and compared to the theoretical ones ($m/z_{\text{theoretical}}$). The difference parameter (mass accuracy; in ppm units) was calculated following Equation (1):

$$\text{Difference} = [(m/z_{\text{theoretical}} - m/z_{\text{measured}})/m/z_{\text{theoretical}}] \times 10^6 \quad (1)$$

The purity of prepared or analyzed compounds (**3**, **5a–g**, and **6a–g**) was verified by HPLC hyphenated with ultraviolet spectrophotometry and high-resolution mass spectrometry (LC-UV/HR-MS) as listed in the Supplementary Materials.

2.1.2. Synthesis of Compounds

- Preparation of ($\pm$)-(oxiran-2-yl)methyl-[1-(3-trifluoromethyl)phenyl]carbamate (**3**)

The synthesis of the compound (**3**) was published in [30], and the procedure is also mentioned in the Supplementary Materials. The yield, M_r , Mp, and R_f as well as ^1H NMR, ^{13}C NMR, ^{19}F NMR, and HPLC-UV/HR-MS (ESI) analyses for a given off-white solid (Figure 4) are also available [30]. Thus, some missing data were estimated within the current research, i.e., IR spectral analysis and purity. The complete characterization of this intermediate is provided in the Supplementary Materials.

- General preparation of carbamate compounds (**5a–g**)

The general synthetic procedures, according to which the proposed carbamate compounds (**5a–g**) were prepared, can be found in [30] and are also included in the Supplementary Materials together with the characteristics of employed basic amines (**4a–g**). The oily compounds **5a–d** and **5f** are newly synthesized within the scope of current research, and their complete spectral investigation and analytical data are provided in the Supplementary Materials.

The synthesis and majority of the spectral as well as analytical characteristics of the molecules **5e** and **5g** are published in [30] and, in addition, they are also included in the Supplementary Materials. Some missing parameters for these derivatives were estimated currently (i.e., R_f values (TLC), IR spectral assignments, and purity) and are provided in the Supplementary Materials as well.

- General preparation of piperazinium chloride salts (**6a–g**)

The general synthetic procedures, according to which the desired piperazinium chloride salts (**6a–g**) were synthesized, can be found in [30], and the details are also given in the Supplementary Materials. The off-white solids **6a–d** and **6f** (Figure 4) are newly prepared within the scope of current research, and their complete spectral analyses, analytical and physicochemical data are provided in the Supplementary Materials as well as in the next sections of this article.

The other off-white solids, **6e** and **6g** (Figure 4), were synthesized and described mostly within the research in [30], and their yields, M_r , M_p , ^1H NMR, ^{13}C NMR, ^{19}F NMR, and HPLC-UV/HR-MS (ESI) are available; therefore, the missing R_f values (TLC) and IR spectral analyses and purity were able to be estimated within the present study. All required descriptors are listed in the Supplementary Materials. In addition, ^1H NMR, ^{13}C NMR, and ^{19}F NMR spectra as well as HPLC-UV/HR-MS analyses of the compounds **6a–g** are provided in the Supplementary Materials (Figures S1–S28).

2.1.3. Determination of Some Physicochemical Properties

- Estimation of electronic properties

The electronic properties of **6a–g** were defined by the values of the logarithms of molar absorption coefficients ($\log \epsilon$) following Lambert–Beer’s law (Equation (S1)). The details regarding employed experimental procedures published in [31,32] are also listed in the Supplementary Materials. The $\log \epsilon$ values for the presently observed absorption maxima (λ) of **6a–g** (i.e., $\lambda_1 = 203.50\text{--}206.00$, $\lambda_{2(\text{Ch-T})} = 238.50\text{--}243.00$ (charge-transfer absorption maximum), and $\lambda_3 = 279.50\text{--}286.50$ nm) are provided in Table 1.

Table 1. The absorption maxima (λ_1 , $\lambda_{2(\text{Ch-T})}$, and λ_3 , in nm) observed in the UV/Vis absorption spectrum from 190 to 820 nm and logarithms of molar absorption coefficients ($\log \epsilon_1$, $\log \epsilon_{2(\text{Ch-T})}$ and $\log \epsilon_3$) of target compounds’ methanolic solutions ($c = 5.0 \times 10^{-5}$ M).

Entry	λ_1 (nm)	$\log \epsilon_1$	$\lambda_{2(\text{Ch-T})}$ (nm)	$\log \epsilon_{2(\text{Ch-T})}$	λ_3 (nm)	$\log \epsilon_3$
6a	203.50	4.53	238.50	4.30	280.00	3.25
6b	203.50	4.52	239.00	4.28	280.50	3.21
6c	204.00	4.24	239.00	4.27	281.00	3.24
6d	204.00	4.48	241.50	4.28	280.50	3.25
6e	206.00	4.55	240.00	4.32	286.50	3.34
6f	205.00	4.51	241.00	4.31	286.50	3.57
6g	204.00	4.47	243.00	4.33	279.50	3.07

- Estimation of lipophilic properties

Lipohydrophilic characteristics of **6a–g** were estimated by reversed-phase high-performance liquid chromatography (RP-HPLC). Details on the measurements and calculation of retention factors (capacity factors; k) according to Equation (S2) can be found in the Supplementary Materials as well as in [31]. The calculations for the extrapolated retention (capacity) factor (k) logarithms for 100% water (isocratic RP-HPLC)— $\log k_w$ parameters (Table 2)—were made from intercepts of linear plots between $\log k$ and φ_M following Equation (2):

$$\log k = \log k_w - S \times \varphi_M \quad (2)$$

where the S parameter is the slope of a regression curve describing the solvent strength of a pure organic solvent, and φ_M is the volume fraction of MeOH [33,34].

The experimental details considering methods for the purity verification of **6a–g** are provided in [31] and the Supplementary Materials (see Tables S1 and S2).

Table 2. Extrapolated $\log k_w$ parameters and S (slope) data (RP-HPLC) of the **6a–g** set together with the calculated values of statistical descriptors (χ^2_{red} , RSS , R , $Adj. R^2$, $RMSE$, F , and $Prob > F$), which defined the linearity between $\log k$ and ϕ_M for a particular molecule. The indication of a significance level of the F -ratio ($Prob > F$ parameter): extremely significant in all cases.

Entry	$\log k_w$	S	$^1 \chi^2_{\text{red}}$	$^2 RSS$	$^3 R$	$^4 Adj. R^2$	$^5 RMSE$	$^6 F$	$^7 Prob > F$
6a	4.1170	4.7782	0.0036	0.0179	0.9945	0.9867	0.0598	447.63	4.37×10^{-6}
6b	4.8419	5.5848	0.0017	0.0066	0.9976	0.9939	0.0408	821.64	8.82×10^{-6}
6c	4.2672	4.9826	0.0040	0.0201	0.9943	0.9863	0.0635	431.66	4.78×10^{-6}
6d	4.8631	5.5062	0.0020	0.0097	0.9977	0.9945	0.0441	1089.80	4.79×10^{-7}
6e	5.0882	5.5840	0.0004	0.0016	0.9997	0.9992	0.0176	7051.83	4.54×10^{-9}
6f	4.9260	5.3504	0.0023	0.0114	0.9972	0.9932	0.0478	876.96	8.23×10^{-7}
6g	5.2184	5.9042	0.0011	0.0056	0.9989	0.9973	0.0335	2180.52	8.51×10^{-8}

¹ χ^2_{red} , reduced chi-square; ² RSS , residual sum of squares; ³ R , correlation coefficient; ⁴ $Adj. R^2$, adjusted coefficient of determination; ⁵ $RMSE$, root mean square error (standard deviation); ⁶ F , Fisher's significance ratio (Fisher's F -test); ⁷ $Prob > F$, probability of obtaining the F -ratio (significance of a whole model).

2.2. Biological Assays

2.2.1. In Vitro Antimycobacterial Evaluation

The activity of **6a–g** and isoniazid (INH) standard drug (Sigma-Aldrich, Darmstadt, Germany) was inspected in vitro against an attenuated *M. tuberculosis* H₃₇R_a ATCC 25177 (*Mtb* H₃₇R_a), *M. kansasii* DSM 44162 (MK DSM), *M. smegmatis* ATCC 700084 (MS), and *M. marinum* CAMP 5644 (MM). The experimental procedures for susceptibility testing of given tuberculous and non-tuberculous mycobacteria are published in [35–42], and the details are also listed in the Supplementary Materials. The estimated MIC values (in μM units), i.e., the lowest concentrations of a compound at which no visible growth of a particular bacteria was observed, can be found in Table 3.

Table 3. The in vitro biological evaluation of the **6a–g** set and isoniazid (INH) reference drug including their activity (MIC values, in μM units) against *M. tuberculosis* H₃₇R_a ATCC 25177 (*Mtb* H₃₇R_a), *M. kansasii* DSM 44162 (MK DSM), *M. smegmatis* ATCC 700084 (MS), and *M. marinum* CAMP 5644 (MM) as well as toxicity in HepG2 cells (IC₅₀ values, in μM units).

Entry	MIC (μM)				IC ₅₀ (μM) HepG2
	<i>Mtb</i> H ₃₇ R _a	MK DSM	MS	MM	
6a	69.58	69.58	139.16	69.58	83.08
6b	33.76	33.76	67.52	33.76	33.10
6c	33.48	66.96	133.92	66.96	49.36
6d	32.36	8.09	64.73	15.12	43.35
6e	3.78	15.13	30.25	15.12	29.39
6f	15.15	15.15	15.15	30.36	54.71
6g	3.63	14.54	14.54	8.09	22.18
INH	58.33	29.16	43.75	29.16	nd

nd = not determined.

2.2.2. In Vitro Cytotoxicity Screening

The potential of **6a–g** to reduce the in vitro viability of the human Caucasian hepatocyte carcinoma (HepG2) cell line population to 50% of its maximum viability was described with inhibitory concentrations (IC₅₀ values, in μM units). The methods for in vitro estimation of IC₅₀ parameters are published in [43,44] and, in addition, are also provided in the Supplementary Materials. The IC₅₀ value for a particular molecule (Table 3) was acquired from a nonlinear regression analysis based on the dependence between a semi-logarithmic plot of incubation concentration and percentage of absorbance (A) value relative to untreated controls [43,44]. These IC₅₀ descriptors were calculated using Microsoft Excel software, version 2016 (Microsoft Corp., Redmont, WA, USA).

2.3. In Silico Evaluation of Synthesized Bases

The PASS Online (prediction of activity spectra for substances) program (Department of Bioinformatics, Laboratory of Structure–Function Based Drug Design, Institute of Biomedical Chemistry, Moscow, the Russian Federation) was used to predict a spectrum of biological activities [45,46] for synthesized bases **5a–g**. The probability that the analyzed compounds might be biologically active was considered and expressed by values of relevant indices (*I*). These *I* values varied from 0.000 to 1.000. The probability that analyzed bases might act as general pump inhibitors (I_{GPI}) and membrane permeability inhibitors (I_{MPI}) as well as drugs for cancer-associated disease treatments (I_{CADT}) was predicted. In addition, the probability of their possible toxic effects was also predicted (Table S3), i.e., the probability of causing bradycardia (I_B), palpitation (I_P), and tachycardia (I_T) as well as prolongation of a QT interval (I_{QTp}).

Due to the fact that the PASS Online predictor processes so-called small molecules (i.e., compounds with $M_r < 900$ –1000) and the predictor's limitation is $M_r < 1250$, it was not possible to perform analyses for the corresponding final compounds (**6a–g**), because no correct simplified molecular-input line-entry system (SMILES) codes were generated. In other words, the interactive environment of this freeware program did not allow for the processing of charged compounds or for the prediction of required descriptors [45,46]. Moreover, INH was not included in the in silico analyses because not all required indices were generated for a given reference drug.

2.4. Calculations and Statistical Analyses

The OriginPro 2019b software, version 9.6.5.169 (OriginLab Corporation, Northampton, MA, USA), was used to calculate particular statistical descriptors of linear regression analyses ($\log k_w$, S , χ^2_{red} , RSS , R , $Adj. R^2$, and $RMSE$) and analyses of variance (ANOVA; F and $Prob > F$), both related to current RP-HPLC experiments (Table 2). Brief definitions of all calculated parameters and significance levels (indicated by stars) as well as several fundamental terms (i.e., principal components (PCs), pretreatment methods, scree plots, eigenvalues (λ_e), circle of correlation, and Pearson's correlation coefficient (r), which are valid for linear regression analyses, ANOVA, and principal component analysis (PCA)) can be found in References [47–51] and the Supplementary Materials.

The XLSTAT software, version 2016.02.28451 (Addinsoft, New York, NY, USA) was employed for PCA [49] to explore properly the relationships between values of estimated analytical and physicochemical descriptors ($m/z_{measured}$, $\log \varepsilon_2$ (Ch–T), and $\log k_w$) as well as $\log (1/MIC)$ (M) and $\log (1/IC_{50})$ (M) parameters derived from both the MIC and IC_{50} data sets. These parameters resulted from in vitro biological (antimycobacterial and cytotoxic) evaluation of **6a–g** (Table 3). In addition, the in silico descriptors (I_{GPI} , I_{MPI} , I_{CADT} , I_B , I_P , I_T and I_{QTp} ; Table S3) of corresponding bases (**5a–g**) were also involved in these analyses.

3. Results and Discussion

3.1. Chemistry

3.1.1. Synthesis of Compounds

In regard to the objectives set, the molecules were synthesized as follows (Figure 4). Lipophilic 3-(trifluoromethyl)phenyl isocyanate (**1**), as a convenient starting compound, was treated with ($\pm$)-(oxiran-2-yl)methanol (**2**) providing white crystalline ($\pm$)-(oxiran-2-yl)methyl-[1-(3-trifluoromethyl)phenyl]carbamate (**3**). The yield of given nucleophilic addition was satisfactory (81%). The opening of an ($\pm$)-(oxiran-2-yl)methyl ring of **3** was made via nucleophilic addition with particular 1-(substituted phenyl)-/1-(dimethylphenyl)piperazines (**4a–g**) available commercially [30,52]. The desired oily carbamates (**5a–g**) were obtained with good yields from 75% (**5f**) to 85% (**5g**).

The bases dissolved in chloroform were converted into required piperazinium chloride salts (**6a–g**). In order to obtain pure target compounds for further analytical, physicochemical, and in vitro biological evaluations, they were crystallized from propan-2-ol twice. Thus,

final substances were achieved with moderate to low yields only, i.e., from 55% (**6g**) to 31% (**6c**).

The molecules **6a–g** were defined comprehensively with appropriate descriptors from IR, ^1H NMR, ^{13}C NMR, and ^{19}F NMR as well as HPLC-UV/HR-MS (ESI) spectral analyses, and these data confirmed the proposed structures. Considering the IR spectra interpretation (Supplementary Materials), the bands from 1740 (**6f**) to 1722 cm^{-1} (**6c**) were observed as typical ones for stretching vibrations, $\nu(\text{C}=\text{O})$. The phenyl moieties were indicated with $\nu(\text{C}=\text{C})$ at approximately 1605 cm^{-1} . The vibrations at approximately 1512 cm^{-1} were observed as $\delta(\text{N}-\text{H})$ was present. The bands between 1245 and 1242 cm^{-1} corresponded to asymmetric stretching of the C–O–C arrangement of bonds. The $=\text{C}-\text{H}$ group afforded in-plane deformation vibrations (δ_{ip}) approximately at 1020 cm^{-1} and, in addition, its out-of-plane deformation vibrations (δ_{oop}) approximately at 845 cm^{-1} were also noticed. In ^1H NMR (Supplementary Materials), the proton signal of a protonated cyclic piperazin-1-ium-1,4-diyl moiety was observed as a singlet in the δ interval from 10.95 (**6b**) to 10.46 ppm (**6g**). The proton signal of an NHCOO group was observed as a singlet from 10.24 (**6a**) to 9.99 ppm (**6c**). Following the ^{13}C NMR spectral data (Supplementary Materials), all carbons were confirmed in agreement with the proposed structures. The signal of fluorine within a CF_3 group was observed from -60.14 (**6b**) to -60.20 ppm (**6c**) in ^{19}F NMR. Heteronuclear coupling of fluorine atoms to ^1H NMR and ^{13}C NMR was also observed (Supplementary Materials). The protonation of only one N atom of piperazin-1-ium-1,4-diyl was observed within the structure of **6a–g** (Figure 4). The finding was supported by HPLC-UV/HR-MS, where particular $[\text{M}+\text{H}]^+$ molecular peaks for these solid molecules were found (Supplementary Materials).

The purity evaluation of all salts was carried out using the LC-UV/HR-MS method. Their satisfactory purity, varying from 97.34% (**6a**) to 98.85% (**6g**; Supplementary Materials), was achieved by repeated crystallization as listed in previous sections.

3.1.2. Determination of Some Physicochemical Properties

- Determination of electronic properties

The UV/Vis absorption spectra of methanolic solutions ($c = 5.0 \times 10^{-5}\text{ M}$) of **6a–g** were measured in the λ interval of 190–820 nm. Three absorption maxima (Table 1) were found for these solutions as follows: $\lambda_1 = 203.50\text{--}206.00$, $\lambda_{2(\text{Ch-T})} = 238.50\text{--}243.00$, and $\lambda_3 = 279.50\text{--}286.50\text{ nm}$. This observation was in full agreement with [53], as the positions of particular absorption bands were typical for (substituted) phenylcarbamic acid derivatives. The observed bands were labeled properly as first local excitation (λ_3), charge-transfer ($\lambda_{2(\text{Ch-T})}$), and second local excitation (λ_1) absorption maxima, respectively.

Almost all substituents attached to the aromate of a 4-(substituted phenyl)piperazin-1-ium-1-yl moiety, which carried non-bonding electrons, caused shifts in the λ_1 and λ_2 as well as λ_3 absorption bands to longer wavelengths. Thus, bathochromic shifts at these absorption maxima were found for the compounds **6b–g** compared to those of **6a**, a molecule containing a 4-phenylpiperazin-1-ium-1-yl group. This trend was in agreement with conclusions already published [54]. In fact, exceptions were observed only for the molecule **6b** and its λ_1 absorption band (equal values were found out) as well as **6g** and its λ_3 absorption band, for which a slight hypsochromic shift was observed (Table 1).

The $\log \epsilon_{2(\text{Ch-T})}$ parameter was the most sensitive to the differences in length, position, electronic, and steric properties of substituent(s) attached to a piperazin-1-ium-1,4-diyl moiety. Thus, when discussing SARs, special consideration was paid to $\log \epsilon_{2(\text{Ch-T})}$. Absorption intensity at this maximum increased slightly for the compounds containing bulky substituents (auxochromes) with strong EW properties (**6e**, **6f**) and molecules with a bulky diphenylmethyl moiety (**6g**). This behavior is called a hyperchromic shift [55]. On the other hand, a hypochromic shift [55] (i.e., decreased values of a given parameter) was observed for mono-substituted derivatives (Table 1) containing a 4'- CH_3 , 4'-F or 4'-Cl substituent (**6b–d**).

- Determination of lipohydrophilic properties

Pathogenic *Mtb* strains possess an external cell wall composed mainly of a covalently attached complex of a branched polysaccharide peptidoglycan, lipopolysaccharides (arabinogalactan, lipoarabinomannan, and lipomannan), and highly lipophilic mycolic acids. These structures have important roles in the growth and integrity maintenance of the cell wall. In addition, *Mtb* has an outer layer composed of an array of glycolipids, lipoglycans, and lipids associated with this wall [56–58]. Thus, the lipophilicity is an important parameter that should definitely be taken into consideration in the design of novel anti-TB compounds to ensure their permeation via the cell wall of *Mtb* [59].

Computational methods (i.e., CLOGP, ALOGP, XLOGP2, or XLOGP3) are frequently used to calculate or predict the lipophilicity of various synthetic or natural antimycobacterial compounds [60–65]. However, it would be questionable if such in silico approaches might be considered accurate in predicting the lipophilic behavior of the compounds **6a–g**. The structure of the molecules investigated in [60–65] allowed for such predictions; however, particular predictors were not designed to evaluate salts, i.e., charged compounds as discussed in [31].

Taking into strong consideration this aspect, the lipohydrophilic features of **6a–g** were investigated by an experimental RP-HPLC technique. The retention behavior of a molecule in a reversed-phase (RP) chromatographic system is in very close connection with its lipophilicity commonly derived from $\log k$ [66]. The C₁₈-functionalized silica gel (stationary phase; SPh) and liquid binary mixtures of methanol (MeOH) in different volume concentrations (60–90%; *v/v*) with 5% increments and water [66] were employed as suitable mobile phases (MPs) in order to estimate these $\log k$ parameters (Tables S1 and S2).

The highest t_r and $\log k$ data were determined for the most lipophilic compounds **6f** ($t_r = 26.5828$ min, $\log k = 1.7516$), **6e** ($t_r = 24.7159$ min, $\log k = 1.7372$), and **6g** ($t_r = 23.1377$ min, $\log k = 1.6913$) in the MPH with a 60% proportion (*v/v*) of the organic modifier. The increase in the amount of MeOH (*v/v*) lowered the values of both t_r and $\log k$ parameters as expected (Tables S1 and S2).

The estimated $\log k$ descriptors for **6a–g**, however, might not be considered universal parameters to adequately express their lipophilicity because of their being very notably influenced by particular chromatographic conditions [67]. The extrapolation of estimated solute retention ($\log k$) to the elution with 100% water ($\log k_w$) was regarded as a much more adequate procedure (Table 2). The impact of lipophilicity, expressed with $\log k_w$, on the in vitro antimycobacterial activity of these compounds might be expected [31,47].

The linear relationships between estimated $\log k$ and φ_M related to particular molecules were statistically extremely significant, as all calculated probabilities of obtaining *F*-ratio ($Prob > F$) values belonged to the interval from 0.0000 to <0.0010 (Table 2). Very acceptable quality, which characterized all suggested linear relationships providing desired extrapolated parameters ($\log k_w$ s), was adjusted with the reduced chi-square (χ^2_{red}) descriptor varying from 0.0004 (**6e**) to 0.0040 (**6c**). Next, the linearity of all observed models was defined with minimal RSS (residual sum of squares) outputs from 0.0016 (**6e**) to 0.0201 (**6c**) as well as with the root mean square error (RMSE; Table 2) found between 0.0176 (**6e**) and 0.0635 (**6c**).

The $\log k_w$ values of **6a–g** ranged from 4.1170 (**6a**) to 5.2184 (**6g**), considering the variably substituted piperazin-1-ium-1,4-diyl moiety. The lipophilicity of a 4'-CH₃ substituted molecule (**6b**; $\log k_w = 4.8419$) was higher than that containing a 4'-F atom (**6c**; $\log k_w = 4.2672$). This finding was in agreement with the calculated values of the hydrophobic substituent constant (π) of both substituents, which were as follows [68]: $\pi_{(4'-CH_3)} = 0.53$ and $\pi_{(4'-F)} = 0.14$.

3.2. Biological Assays

3.2.1. In Vitro Antimycobacterial Evaluation

The efficiency of **6a–g** and the INH reference drug was inspected in vitro against *Mtb* H₃₇R_a, MK DSM, MS as well as *M. marinum* (Table 3) and described with the MIC parameter (in μ M units) [39,40]. Tuberculous *Mtb* H₃₇R_v and H₃₇R_a were used to confirm (or refuse) if

the newly synthesized and traditional anti-TB drugs showed in vitro activity. The question of whether H₃₇R_a might “substitute in some manner” a highly pathogenic H₃₇R_v or clinical tuberculous mycobacterial strains was answered clearly by Heinrichs et al. (2018). The authors concluded that the H₃₇R_a strain was as equally useful as the H₃₇R_v one regarding responses to in vitro screened anti-TB drugs. Moreover, the biosafety requirements concerning H₃₇R_a were much more convenient compared to those of H₃₇R_v [69].

The most powerful antimycobacterials were 1-[2-hydroxypropyl-((3-trifluoromethyl)phenyl)carbamoyloxy]-4-(3,4-dichlorophenyl)piperazin-1-ium chloride (**6e**) and 1-[2-hydroxypropyl-((3-trifluoromethyl)phenyl)carbamoyloxy]-4-(4-diphenylmethyl)piperazin-1-ium chloride (**6g**) showing MIC < 3.80 µM against *Mtb* H₃₇R_a (Table 3). These compounds were more active than INH (MIC = 58.33 µM).

Considering previous conclusions [70–72], it might be stated that a 3-CF₃-phenylcarbamoyloxy fragment notably improved the in vitro activity of such substituted compounds against a given *mycobacterium* compared to the efficiency of the derivatives bearing an 2-/3-/4-alkoxyphenylcarbamoyloxy moiety (alkoxy = methoxy or ethoxy).

In addition, both 1-[2-hydroxypropyl-((3-trifluoromethyl)phenyl)carbamoyloxy]-4-(4-chlorophenyl)piperazin-1-ium chloride (**6d**) and **6e** were the most effective against MK DSM with MIC = 8.09 µM (**6d**) and 15.13 µM (**6e**), respectively. Their potency was higher than that of INH (MIC = 29.16 µM; Table 3). The molecule **6g** was the most active against both *MS* (MIC = 14.54 µM) and *MM* (MIC = 8.09 µM). This substance was even more promising than INH (MIC = 43.75 and 29.16 µM, respectively) as listed (Table 3).

The presence of an EW (**6c–f**) or even an electron-donating (ED; **6b**) substituent attached to a phenyl ring seemed to be more beneficial in structural variation in order to potentiate in vitro antimycobacterial activity than classical bioisosteric replacement of this substituent with hydrogen (**6a**). Bulky lipophilic diphenylmethyl moiety (**6g**) improved efficiency, especially against *Mtb* H₃₇R_a and *MM* (Table 3).

The compounds **6a–g** fought in applsci-1501121 vitro tested non-tuberculous mycobacteria more effectively than the substances containing an 2-/3-/4-alkoxyphenylcarbamoyloxy moiety (alkoxy = methoxy or ethoxy) despite the presence of a lipophilic 4'-F atom or 3'-CF₃ group within their salt-forming part [19].

3.2.2. In Vitro Cytotoxicity Screening

Another goal of this research was to set in vitro the impact of **6a–g** on a liver cancer HepG2 cell line. The model was chosen since antimycobacterial agents have been included within a group of the top five pharmacodynamic classes associated with the highest reporting odds ratio (ROR) score. In attempt to set this ROR score, the frequency of hepatotoxicity was estimated at both the group and individual drug levels. The ROR score index describes the measure of such undesirable phenomenon [73]. The highest ROR scores with the highest risk of drug-induced liver injury were observed for the compounds belonging to antineoplastic, antiviral, analgesic, antibiotic, and antimycobacterial classes.

The molecules **6a–g** were highly lipophilic as their log *k_w* values from 4.1170 (**6a**) to 5.2184 (**6g**) indicated (Table 2). Potential disadvantages of highly lipophilic bedaquiline (**BDQ**), a very effective antimycobacterial agent (Figure 5), include the inhibition of a human Ether-à-go-go-related gene (hERG) potassium channel due to the concurrent risk of cardiac toxicity, possible phospholipidosis, or hepatic toxicity. The shortening or lengthening of its dimethylaminoethyl sidechain did not improve antimycobacterial activity, but increased cytotoxicity was observed as the lipophilicity of synthesized **BDQ** derivatives increased [74,75].

Figure 5. Chemical structure of bedaquiline (BDQ) and its structural modifications that lead to more cytotoxic derivatives or to molecules showing undesirable side effects.

The current nonlinear regression analyses explored the relationships between a semi-logarithmic plot of incubation concentration and percentages of absorbance relative to untreated controls, thus providing IC_{50} values [63]. These IC_{50} parameters (in μM units) describing the cytotoxic properties of **6a–g** varied from 22.18 (**6g**) to 83.08 μM (**6a**) as listed in Table 3. The most effective compounds (**6g** and **6e**) against *Mtb* H₃₇R_a showed IC_{50} = 22.18 (**6g**) and 29.39 μM (**6e**). If accepting IC_{50} > 25.00 μM as a “very mild” IC_{50} criterion characterizing low toxic derivatives [76], the cytotoxicity of **6e** might be “theoretically acceptable”. However, Ambrozkiewicz et al. (2020) stated that the molecules with $IC_{50} \leq 100.00$ μM were relatively cytotoxic [77]. The INH standard drug showed a very limited ability to reduce the population of HepG2 liver cancer cells (IC_{50} > 1000 μM) [63].

The limited safety of effective antimycobacterial agents due to the relatively low IC_{50} values was the reason for not progressing further. Poce et al. (2019) stated that molecules with $IC_{50} \geq 40.00$ μM were safe [78]. Following the conclusions published in [76,78], both **6d** (IC_{50} = 43.35 μM) and 1-[hydroxypropyl-[(3-trifluoromethyl)phenyl]carbamoyloxy]-4-(3-trifluoromethylphenyl)piperazin-1-ium chloride (**6f**; IC_{50} = 54.71 μM) might be “theoretically safe”. In regard to confirming or refusing the validity of statements about the cytotoxic properties of the compounds **6d–g** based on the conclusions published in [76,78], previous in vitro investigations [30,79,80] also contributed to the current quest.

Bouz et al. (2021) evaluated the in vitro cytotoxic properties of a tamoxifen (TMX) reference drug against the HepG2 cell line. The authors described the efficiency of TMX with IC_{50} = 19.56 μM [63]. Other anticancer drugs, such as afatinib, 5-fluorouracil, and sorafenib, showed IC_{50} = 5.40, 7.91, and 9.18 μM [79,80], respectively.

The compound **6g** also efficiently inhibited the proliferation in vitro of a human monocytic leukemia THP-1 cell line showing IC_{50} = 5.91 μM as the concentration, which inhibited 50% of the proliferation. Moreover, lower cytotoxic activity of a cisplatin standard drug was even observed (IC_{50} = 13.65 μM) in those experiments [30]. Thus, both the molecules **6e** and **6g** were suggested as suitable structural platforms for the development of promising anticancer agents, but their eventual use in pharmacotherapeutic interventions as anti-TB drugs has to be considered.

The current “definitive” conclusion was supported by calculations of selectivity index (SI) parameters. The ratio between particular IC_{50} values (in μM units) of the molecules **6a–g** and their MIC parameters (in μM units) estimated against *Mtb* H₃₇R_a, MK DSM, MS, and MM [81], respectively, was defined as SI (Table S4). The highest SI values were calculated for **6e** (SI = 7.78) and **6g** (SI = 6.11) as the ratio of their activity against HepG2 liver cancer cells and efficiency against *Mtb* H₃₇R_a as well as for **6d** (SI = 5.36) as the ratio of its activity against these cancer cells and efficiency against MK DSM.

Very thin line was found between in vitro cytotoxic and antimycobacterial properties of both **6b** and **6c** (Table 3). The calculated SI values preliminarily indicated that the anti-MS activity of these compounds might be the consequence of their cytotoxicity, as SI = 0.49 (**6b**) and 0.37 (**6c**) as shown in Table S4. Secrist et al. (2001) determined that

if $SI > 10.00$, a drug candidate might be regarded as safe granting sufficient difference between (antimycobacterially) efficient and cytotoxic concentrations [81]. Overall, current experiments clearly indicate safety concerns connected with the **6a–g** set.

3.3. In Silico Evaluation of Synthesized Bases

Regarding the analyses of prepared bases (**5a–g**), the PASS Online program [45,46] predicted the probability of their acting as general pump inhibitors (I_{GPI}) and membrane permeability inhibitors (I_{MPI}) as well as drugs for the treatment of cancer-associated diseases (I_{CADT}). In addition, the possible toxic effects of these bases on a cardiovascular system (i.e., probability of causing bradycardia (I_B), palpitation (I_P), and tachycardia (I_T) as well as prolongation of a QT interval in the heart (I_{QTp})) were also predicted (Table S3). The criteria for prediction of a specific activity are provided in the Supplementary Materials.

BDQ inhibits the synthesis of ATP in mycobacteria. The substance interferes with the activity of an F_1F_0 ATP synthase enzyme by inhibiting its proton pump, which is critical for the generation of membrane potential. This synthase is one of the enzymes considered essential for meeting the energy requirements of both the proliferating aerobic and hypoxic dormant stages of the life cycle for a variety of mycobacterial strains. ATP levels are highly reduced within bacterial cells upon the action of **BDQ** [82,83].

The highest probability to act as general pump inhibitors was calculated for the compounds containing one or two phenyl rings substituted with hydrogens only (Table S3), i.e., **5g** ($I_{GPI} = 0.707$) and **5a** ($I_{GPI} = 0.613$). The presence of other substituents provided lower values regardless of their ED (**5b**) or EW (**5c–f**) properties.

The pathogenic properties of *Mtb* are based on thwarting the innate defense systems of a macrophage host cell. Virulence factors of *Mtb* disrupt physiological host cell signaling. The destruction of macrophages starts when the pathogen enters a cell, affecting (destroying) a variety of host signal transduction pathways. Moreover, the surface of these cells is remodulated as a consequence of *Mtb* action via altering the expression of the molecules, which are crucial for full activation of an adaptive immune response [84].

Membrane permeability inhibitors could more or less block this process. The predicted I_{MPI} parameter for synthesized bases varied from 0.457 (**5g**) to 0.555 (**5d**). It seemed that the probability of their blocking activity was related to a precise selection of a substituent owning specific electronic and steric properties. The 4'-Cl substitution was the most favorable (**5d**), the lipophilicity of a given atom might be described by $\pi_{4'-Cl} = 0.71$ and the EW effect with a Hammett substituent constant σ ($\sigma_{4'-Cl} = 0.23$). The values set for hydrogen were as follows [68]: $\pi_H = 0.00$, $\sigma_H = 0.00$.

Special attention should be paid to the molecule **5g** (as well as its antimycobacterially effective salt **6g**), as $I_T = 0.605$ and $I_{QTp} = 0.640$. The issue of polymorphic ventricular tachycardia and QT interval prolongation was also observed for **BDQ**. If the interval is prolonged, polymorphic ventricular tachycardia and/or “torsades de pointes” can occur. These dysfunctions may precipitate ventricular fibrillation resulting in sudden death. Fortunately, severe QT interval prolongation is an infrequent cause of **BDQ** interruption despite the frequent use of concomitant drugs that also prolong the QT interval [85,86].

3.4. Structure-Activity Relationships

The PCA approach was employed to explore the relationships between observed values of analytical and physicochemical parameters for the **6a–g** set (m/z_{measured} , $\log \varepsilon_2$ (Ch-T), and $\log k_w$), in silico descriptors (I_{GPI} , I_{MPI} , I_{CADT} , I_B , I_P , I_T , and I_{QTp}) related to **5a–g**, and the calculated data based on in vitro biological screening of the compounds **6a–g**, i.e., $\log (1/\text{MIC (M)})$ and $\log (1/\text{IC}_{50} \text{ (M)})$, respectively. Firstly, all analyzed indices were precisely transformed using several data pretreatment techniques [87–89]. Secondly, a scree plot was constructed to correctly set a number of relevant principal components (PCs). The scree plot described adequately the relationships between calculated eigenvalues (λ_e) and number of PCs. The Kaiser–Guttman rule [50], as the most common stopping rule in PCA, aimed to limit the number of PCs—only PCs with $\lambda_e > 1.00$ were considered.

The PC 1, PC 2, and PC 3, which indicated the mathematical possibility of the existence of “real” parameters, accounted for 86.84% of the total variance, and their contributions were as follows: PC 1 (64.85%, $\lambda_e = 9.73$), PC 2 (15.00%, $\lambda_e = 2.25$), and PC 3 (6.99%, $\lambda_e = 1.05$). The relationship between PC 1 and PC 2 allowed for the distribution of **6a–g** into several subcategories. The sum of these PCs expressed the majority of the data variability (79.85% in total), as shown in Figure 6a,b.

Figure 6. Graphical presentation of SARs for the **6a–g** set: (a) two-dimensional (2D) score plot, which shows the (i) principal component 1 (PC 1) and 2 (PC 2) scores; (ii) loadings of particular variables; (b) 2D mapping of loadings of variables, which indicated their (i) positions within a circle of correlation; (ii) relationships with both PC 1 and PC 2. The labeling of analyzed variables was as follows: A (vector related to the m/z_{measured} variable), B ($\log \varepsilon_{2(\text{Ch-T})}$), C ($\log k_w$), I_{GPI} (connected with the I_{GPI} variable), I_{MPI} (I_{MPI}), I_{CADT} (I_{CADT}), I_{B} (I_{B}), I_{P} (I_{P}), I_{T} (I_{T}), I_{QTP} (I_{QTP}), and 1 (vector constructed on the $\log (1/\text{MIC} (\text{M}))$ values. These MIC parameters resulted from the in vitro screening of **6a–g** against *Mtb* H₃₇R_a), 2 (MK DSM), 3 (MS), 4 (MM), and 5 (vector constructed on the $\log (1/\text{IC}_{50} (\text{M}))$ values. These IC_{50} values resulted from the in vitro screening of **6a–g** against the HepG2 cell line).

The PC 1 served as a suitable component to separate quite clearly the antimycobacterially active and inactive derivatives. The subgroup defined by $\text{PC } 1 \leq -0.286$ included **6e–g**, which were relatively effective ($\text{MIC} \leq 30.36 \mu\text{M}$) against all in vitro screened mycobacteria. The substances with $\text{MIC} \geq 64.73 \mu\text{M}$ against at least one mycobacterial strain (**6a–d**) were described with $\text{PC } 1 \geq 0.481$. The lower PC 1 descriptor indicated more active antimycobacterial compound. On the other hand, higher PC 1 parameter was connected with lower activity of a derivative. The combination of both negative PC 1 and PC 2 (Figure 6a,b) can be used to characterize the most active molecule, **6g** ($\text{PC } 1 = -6.365$ and $\text{PC } 2 = -1.817$). The $\text{PC } 1 \geq 2.988$ together with $\text{PC } 2 \geq -1.468$ defined antimycobacterially inactive compounds (**6a** and **6c**). Other differences in the compounds’ properties mirrored in both PC 1 and PC 2 as well as other PCs are provided in the Supplementary Materials (see Table S5).

The distance between particular variables onto a plane and circle of correlation (Figure 6a,b) expressed the quality of their 2D representation. The loadings of given variables (i.e., observed and extrapolated analytical and physicochemical descriptors, the data resulted from in vitro biological evaluations as well as parameters from the PASS Online predictor) defined the contribution (“measure”) of each original variable to the chosen PCs [51]. These loadings are represented by color-coded vectors labeled following their position in a 2D score plot (Figure 6a).

The color-coded vectors were also labeled according to their position in a circle of correlation (Figure 6b). The proper indication of variables with letters A–C or digits 1–5 is provided in the Supplementary Materials. The cycle was characterized with a radius = 1 (absolute value). The visualization based on such relationships provided the sharpest angles between some pairs of vectors, i.e., A and 5, C and 3, 1 and 5, 3 and 4, I_{QTp} and I_{GPI} as well as I_B and I_{CADT} .

Pearson's correlation coefficient (r) values indicated relatively satisfactory correlation between m/z_{measured} (vector 1) of the compounds **6a–g** and their $\log k_w$ (C; $r = 0.8450$), $\log \varepsilon_{2(\text{Ch-T})}$ (B; $r = 0.7959$), in vitro activity against MS (3; $r = 0.9431$), *Mtb* H₃₇R_a (1; $r = 0.9240$) as well as MM (4; $r = 0.8141$). The moderate correlation was observed between m/z_{measured} and toxicity in the HepG2 cell line (5; $r = 0.6681$).

Absorption intensity increased slightly for the compounds containing bulky substituents with strong EW properties (**6e** and **6f**) and molecules with a bulky diphenylmethyl moiety (**6g**). This behavior was connected with increased efficiency, especially against *Mtb* H₃₇R_a (1; $r = 0.7909$) and MS (3; $r = 0.7798$). The correlation between $\log \varepsilon_{2(\text{Ch-T})}$ (B) and $\log k_w$ was weaker (C; $r = 0.5896$). The increased $\log \varepsilon_{2(\text{Ch-T})}$ parameters of analyzed salts increased the probability for their bases to cause tachycardia ($r = 0.7182$). The prolongation of a QT interval was less probable ($r = 0.5260$).

The activity of **6a–g** against all in vitro tested mycobacteria was quite strongly associated with their lipohydrophilic properties. In more detail, the increase in lipophilicity provided higher efficiency against MM ($r = 0.9128$), MS ($r = 0.8650$), and MK DSM ($r = 0.8411$) as well as *Mtb* H₃₇R_a ($r = 0.8199$). It should be stated that both highly lipophilic adamantyl and phenyl moieties in a structure of “similar” compounds did not guarantee particularly high in vitro antimycobacterial activity. The molecule **MOG-1** (Figure 7) was more than two-fold less efficient against *Mtb* H₃₇R_v compared to its regioisomer [90].

Figure 7. The adamantyl group containing compound **MOG-1** was less efficient in vitro against *Mycobacterium tuberculosis* H₃₇R_v compared to its regioisomer. (* = chiral center).

The good news was that a higher $\log k_w$ of **6a–g** might be connected with a notably lower probability of palpitation issues, which could eventually be caused by their bases (**5a–g**; $r = -0.8623$). In addition, the salts would cause neither significant tachycardia ($r = 0.6040$) nor prolongation of a QT interval of the heart ($r = 0.4478$).

The increase in lipophilicity of the tested compounds provided a stronger potential to reduce the in vitro viability of the HepG2 cell population to 50% of its maximum viability ($r = 0.8320$). Efficient anti-MM and anti-*Mtb* H₃₇R_a compounds were effective cytotoxic agents ($r = 0.8138$ and 0.7998 , respectively). The correlation between their anti-MS or anti-MK DSM activity and cytotoxicity was weaker ($r = 0.5820$ and 0.5209 , respectively).

The performed PCA also indicated that similar factors might influence mechanism(s) of action of analyzed molecules (**6a–g**) against particular pairs of mycobacteria as follows: *Mtb* H₃₇R_a and MS ($r = 0.8098$), *Mtb* H₃₇R_a and MM ($r = 0.7980$), MK DSM and MM ($r = 0.8589$) as well as MS and MM ($r = 0.7458$).

4. Conclusions

Various substituents and, thus, different analytical and physicochemical properties of hybrid molecules containing an *N*-(substituted phenyl)-/*N*-diphenylmethylpiperazine privileged scaffold allowed for the modulation of their in vitro activity against several tuberculous and nontuberculous mycobacteria as well as a particular cancer cell line (HepG2).

The absorption intensity increased slightly for the compounds containing bulky substituents with strong EW properties (**6e** and **6f**) as well as the molecule with a bulky diphenylmethyl moiety (**6g**). This behavior strongly influenced the increase in efficiency against both *Mtb* H₃₇R_a ($r = 0.7909$) and *MS* ($r = 0.7798$). The increased lipophilicity of analyzed salts led to higher efficiency against *MM* ($r = 0.9128$), *MS* ($r = 0.8650$), and *MK DSM* ($r = 0.8411$) as well as *Mtb* H₃₇R_a ($r = 0.8199$). Higher lipophilicity of **6a–g** provided their stronger ability to reduce the in vitro viability of HepG2 cell populations to 50% of its maximum viability ($r = 0.8320$). The efficient anti-*Mtb* H₃₇R_a and anti-*MM* compounds (**6e** and **6g**) reduced in vitro the viability of given cells most effectively ($IC_{50} = 22.18$ and $29.39 \mu M$, respectively). The cytotoxic properties of all tested hybrid molecules were not connected significantly with their activity against *MS* ($r = 0.5820$) or *MK DSM* ($r = 0.5209$).

The calculated selectivity index (SI) values for the entire **6a–g** set ($SI < 10.00$) might be considered direct proof that eventual treatment of patients suffering from mycobacterial infections with the analyzed compounds or structurally similar drugs has to be conducted with care. These SI parameters clearly prevented the conclusion that the synthesized molecules, **6a–g**, were safe. In addition, current calculations indicated that risk of tachycardia or QT interval prolongation due to the activity of a basic form of **6g** could not be completely excluded. The proper selection of the substituents attached to the fundamental structural framework of screened substances will be decisive for the development of effective antimycobacterial derivatives with a (notably) weakened ability to reduce the survival and growth of cancer cells or efficient anticancer agents.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app12010300/s1>, Table S1: Retention times t_r and lipophilicity indices $\log k$ (RP-HPLC) of **6a–g** determined in the mobile phases with a various volume ratio (v/v) of a methanol (MeOH) organic modifier and water, i.e., from 60:40 to 70:30; Table S2: Retention times t_r and lipophilicity indices $\log k$ (RP-HPLC) of **6a–g** determined in the mobile phases with a various volume ratio (v/v) of a methanol (MeOH) organic modifier and water, i.e., from 75:25 to 90:10; Table S3: The prediction of probability for the bases (**5a–g**) to act as general pump inhibitors (I_{GPI}) and membrane permeability inhibitors (I_{MPI}) as well as drugs for the treatment of cancer-associated diseases (I_{CADT}). The calculation of probability that these molecules might cause bradycardia (I_B), palpitation (I_P), tachycardia (I_T), and prolongation of a QT interval (I_{QTp}); Table S4: The selectivity index (SI) values of in vitro evaluated compounds **6a–g**, which were calculated as $SI = IC_{50}$ (in μM units)/MIC (in μM units). The IC_{50} s indicated toxicity in human liver hepatocellular carcinoma (HepG2) cells, the MICs were determined against *M. tuberculosis* H₃₇R_a ATCC 25177 (*Mtb* H₃₇R_a), *M. kansasii* DSM 44162 (*MK DSM*), *M. smegmatis* ATCC 700084 (*MS*), and *M. marinum* CAMP 5644 (*MM*); Table S5: The contribution of evaluated variables (in percentages) to particular principal components (PCs; PC 1–6); Figures S1–S28. ¹H NMR, ¹³C NMR, and ¹⁹F NMR spectra as well as HPLC-UV/HR-MS analyses of the compounds **6a–g**.

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