Combinatorially-generated library of 6-fluoroquinolone analogs as potential novel antitubercular agents: a chemometric and molecular modeling assessment

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Abstract The virtual combinatorial chemistry approach as a methodology for generating chemical libraries of structurally-similar analogs in a virtual environment was employed for building a general mixed virtual combinatorial library with a total of 53,871 6-FQ structural analogs, introducing the real synthetic pathways of three well known 6-FQ inhibitors. The druggability properties of the generated combinatorial 6-FQs were assessed using an *in-house* developed drug-likeness filter integrating the Lipinski/Veber rule-sets. The compounds recognized as drug-like were used as an external set for prediction of the biological activity values using a neural-networks (NN) model based on an experimentally-determined set of active 6-FQs. Furthermore, a subset of compounds was extracted from the pool of drug-like 6-FQs, with predicted biological activity, and subsequently used in virtual screening (VS) campaign combining pharmacophore modeling and molecular docking studies. This complex scheme, a powerful combination of chemometric and molecular modeling approaches provided novel QSAR guidelines that could aid in the further lead development of 6-FQs agents.

Keywords Antibacterial agents · CombiChem · DNA gyrase · Fluoroquinolones · Molecular docking · Pharmacophore modeling · QSAR · Tuberculosis

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Abbreviations

TB	Tuberculosis
ATP	Adenosine triphosphate
MIC	Minimal Inhibitory Concentration
6-FQs	6-Fluoroquinolones
SAR	Structure-Activity Relationships
QSAR	Quantitative Structure-Activity Relationships
CombiChem	Combinatorial Chemistry
SSS	Substructure Search
NN	Neural-Networks
KANN	Kohonen Artificial Neural Networks
CP ANN	Counter-Propagation Artificial Neural Networks
GHA	Global Hypothetical Activity
LBP	Ligand-Based Pharmacophore
SBP	Structure-Based Pharmacophore
VS	Virtual Screening

Introduction

Tuberculosis (TB), the ingeniously transferable bacterial infection, is still one of the global health concerns [1]. *Mycobacterium tuberculosis*, the accountant agent of tuberculosis, is a resistful pathogen microorganism responsible for infecting about one third (two billion people) of the human population and in the process causing around two million death cases each year worldwide (World Health Organization, 2003) [2]. TB is mainly caused by the pathogen *M. tuberculosis*, but in some cases the microorganisms such as *M. fortuitum*, *M. smegmatis* and *M. avium-intracellulare* complex (MAC) can also be involved in the disease development [3–5].

The tuberculosis treatment is mainly chemotherapeutically based, with the whole therapy requiring between 6 to 9 months or even longer to be successful. The problem of drug resistance and the continuous onset of multidrug resistant lethal TB strains in most cases are attributed to the potential toxicity of the chemotherapeutics, the durability of the whole treatment, as well as frequent poor patient compliance to the therapy regimen. The increased development of resistant TB mutants is one of the additional challenging factors to stimulate the design of novel chemotherapeutic agents which will be effective against resistant *Mycobacteria* [6].

One of the validated and well known molecular targets of quinolone antibiotics in *Mycobacteria* species is the DNA gyrase which belongs to the topoisomerase group of enzymes [7]. This enzyme catalyzes the ATP dependent process of introduction of negative supercoils into closed circular DNA as well as the relaxation of the supercoiled DNA molecule (ATP-independent catalysis) [8, 9]. DNA gyrase forms a functional heterodimer A_2B_2 consisting of two major subunits, GyrA and GyrB. The GyrA subunit is responsible for the process of breakage and reunion of the double-stranded DNA, i.e., activation of the process of DNA replication and elongation and together with GyrB is involved in the maintaining of the topological state of DNA molecule [10, 11]. Another structurally-similar enzyme which belongs to the topoisomerase group is the DNA topoisomerase IV. Like the DNA gyrase, this bacterial enzyme (a paralogue of DNA gyrase) also forms a functional heterodimer consisting of two subunits ParC and ParE (homologues of the GyrA and GyrB, respectively). Recent structural studies revealed that the quinolone antibiotics establish an interaction with the DNA breakage-reunion domain of the DNA gyrase and topoisomerase IV, stabilizing the covalent topoisomerase/DNA cleavage complex, leading to a blockade of DNA replication [12].

In the last few decades, tuberculosis chemotherapy was mainly based on the active agents belonging to two main categories of antibiotics, the bacterial cell wall inhibitors (isoniazide, ethambutol) and bacterial nucleic acid synthesis inhibitors (quinolones, rifampicin) [13]. The second category of antibiotics, especially the quinolone chemical class of chemotherapeutics is increasingly gaining importance in

targeting *Mycobacteria*, because of their effective, strong and invasive mechanism of action. Fluoroquinolones belong to the quinolone's class of DNA gyrase inhibitors which have a fluorine atom attached to the main scaffold at the 6 position (Fig. 1) [14]. The mechanism of bactericidal action is based on the inhibition of the bacterial DNA synthesis process through a scission of the natural mycobacterial DNA molecule leading to a topological stress of DNA and bacterial cell death [15].

The structure-activity relationships (SAR) studies showed that the main quinolone core (1,4-dihydro-4-oxo-3-pyridinecarboxylic acid moiety) is of most significant importance for the anti-mycobacterial activity. Furthermore, unlike the cyclopropyl group at position 1 which is apparently optimal for biological activity, each substitution at positions 2, 3, and 4 will result in a significant loss of biological activity. Substitutions at positions 5 and 8 of the main quinolone core interfere with the required planarity of the system. Hydrogen and amino groups have been a good replacement for the fluorine atom at position 6 leading to an improved in vitro activity, but such modifications are not always followed by an improved in vivo activity. The substitutions at position 7 of the main scaffold are of significant importance for the biological activity as this position directly interacts with the DNA gyrase (5- or 6-membered N-hetero systems such as aminopyrrolidines and piperazines are optimal for activity) [16]. Today, the use of targeted synthetically-feasible chemical libraries significantly enhanced the hit identification as well as lead optimization phase leading to favorable novel drug candidates. Similarly, quantitative-structure activity relationship (QSAR) models proved to be useful in silico tools for the rationalization of the experimental SAR properties in the form of quantitative mathematical models which can be subsequently used for an efficient prediction of biological activity values for novel as well as unknown compounds. These tools can significantly enhance and enrich the screening process of the chemical libraries under investigation [17]. Furthermore, the three-dimensional assessment and screening of the generated molecules for optimal interaction with the binding site is of significant importance in the drug development processes [18]. For instance, ligand-based drug design approaches, such as pharmacophore modeling, which are taking into account the spatial orientation

Fig. 1 Generic structure of 6-fluoroquinolone antibiotics

of the ligand's functional groups and scaffold shape complementarity can be utilized for the construction of predictive pharmacophore models which can be more effective for virtual screening (VS). Such a model based on the similarity of the pharmacophoric features is particularly useful, if it is able to identify (recognize) active compounds among a pool of inactive molecules. Moreover, the availability of three-dimensional (3D) structure of the protein-ligand complex of interest enables the implementation of the structure-based approaches especially molecular-docking calculations of ligands into the defined binding site of the protein and the investigation of ligand-protein molecular interactions [19, 20].

The present study introduces an effective methodology for the in silico generation of novel unknown 6-fluoroquinolone (6-FQ) analogs using a combinatorial chemistry approach coupled with the prediction of values of biological activity by employing a previously derived neural-networks (NN) [21]. Furthermore, a three-dimensional pharmacophore approach and molecular docking calculations were used to assess the generated virtual library utilizing the available experimental data to select the most promising drug-like compounds. This scheme – a powerful combination of the chemometric and VS tools – is aimed to establish new possible SAR guidelines and trends in 6-fluoroquinolone optimization, which could be of importance in the on-going antibacterial lead discovery programs (Fig. 2).

Fig. 2 The overall workflow depicting different stages of the performed chemometric and molecular modeling study

Computational details

Virtual combinatorial generation

One of the most popular strategies in modern drug design is the virtual synthesis of analogs of existing compounds for which the bioavailability and toxicity studies are already performed and have displayed activity and potency in human therapy. The implementation of SAR-based scaffold modifications to obtain novel molecules for the target under investigation is a well-known and advantageous strategy for analog design. There are several software tools available integrating the combinatorial algorithm for in silico virtual combinatorial generation. *CombiChem* add-on modul available in ChemBioOffice Ultra was used for in silico generation of 6-fluoroquinolone structural analogs [22]. For this purpose we employed the original synthetic pathways of three well known 6-FQ antibiotics: ciprofloxacin, moxifloxacin, and ofloxacin [23–25]. Using a building-blocks commercial dataset (Bionet Fragment Library of 6995 “Rule of 3” filtered lead-like fragments) [26], and SAR-based structural modifications at positions 1 and 7 of the main 6-FQ scaffold, we built six different subsets of combinatorially-generated 6-fluoroquinolone analogs.

The virtual environment enables a rational simplification of the real synthetic reactions taking into account only the crucial synthetic steps for derivation of the final product (structurally-similar 6-FQ analogs). Such a rational virtual simplification resulted in two-steps virtual synthetic pathways for ciprofloxacin and moxifloxacin (yielding R_1 -monosubstituted intermediate product, as well as all possible R_1, R_7 -disubstituted 6-FQ analogs as final products), and one-step virtual synthetic pathway for ofloxacin (obtaining all possible R_7 -monosubstituted analogs as final products). Taking into account that position 7 of the main 6-FQ moiety directly interacts with the DNA gyrase and is of significant importance for activity [16], additionally we introduced several different substituents at this position of the main scaffold with non-amine attachment points. These substructural modifications enable a total of six virtual combinatorial synthetic mechanisms to be defined (Fig. 3):

- (1) 7-amino substituted ciprofloxacin's structural analogs.
- (2) 7-non-amino substituted ciprofloxacin's structural analogs.
- (3) 7-amino substituted moxifloxacin's structural analogs.
- (4) 7-non-amino substituted moxifloxacin's structural analogs.
- (5) 7-amino substituted ofloxacin's structural analogs.
- (6) 7-non-amino substituted ofloxacin's structural analogs.

Fragments selection for combinatorial enumeration

A building-blocks commercial library of 6995 substructural fragments was used as a source for the fragment selection for combinatorial enumeration [26]. Each fragment in such a library is defined by the “rule of 3” ($MW \leq 300$; $nHBD$, $nHBA$, $nRB \leq 3$), where MW is the molecular weight, while $nHBD$, $nHBA$, and nRB , are number of hydrogen bond donors, number of hydrogen bond acceptors, and number of rotatable bonds, respectively [27]. Fragments selection was performed by employing the substructure search (SSS) algorithm. The selection procedure resulted in the extraction of all possible substructural fragments (primary amines and secondary amines/non-amines) defined by the virtual synthetic pathways. Substructural fragmental subsets selected by the SSS algorithm for each virtual synthetic pathway were visually inspected for the presence of possible unwanted species (salt forms, mixtures, charged forms). These substructural fragments were excluded from the combinatorial procedure implementing a simple Boolean filtering algorithm (Y/N (Yes/No)). Furthermore, all substructural fragments containing more than one attachment point (two or more amino groups) were eliminated. The rest of the substructural fragments with (Y) output obtained using this filtering procedure (described in details in Table 1 for each virtual combinatorial definition), were used as a starting point for combinatorial enumeration.

Combinatorial enumeration

The combinatorial enumeration procedure was performed by implementing a standard methodology [28] for generating structural analogs in virtual environment (*CombiChem*) [22]. This procedure of statistical non-repetitive fragmental permutation of the selected building-blocks subsets (R_1 : primary amines and R_7 : secondary amines/non-amines)) in previously defined variable positions (1 and 7) within the main 6-FQ scaffold generated all possible virtual 1,7 substituted 6-FQ structural analogs. The total number of analogs (ρ_{tot}) obtained using this methodology (Table 1), mathematically can be estimated as a multiplication product between the total number of selected substructural fragments permutating at position 1 (R_1 : primary amines, N_i) and total number of selected substructural fragments permutating at position 7 (R_7 : secondary amines/non-amines, M_j) within the main 6-FQ core, using the following equation (Eq. 1) [29]:

$$\rho_{tot} = N_i \times M_j. \quad (1)$$

The obtained products ($\rho_{tot}=53.871$) belong to three main categories of 6-FQ analogs: ciprofloxacin analogs (7-amino substituted analogs (12.296), 7-non-amino substituted analogs (21.965)), moxifloxacin analogs (7-amino substituted

A) Virtual synthetic pathway for combinatorial enumeration of ciprofloxacin analogs:

B) Virtual synthetic pathway for combinatorial enumeration of moxifloxacin analogs:

C) Virtual synthetic pathway for combinatorial enumeration of ofloxacin analogs:

Fig. 3 Generic virtual synthetic pathways for combinatorial enumeration of three different 6-FQs: (A) ciprofloxacin analogs (CIP- N_i - M_j , CIP'- N_i - M_j), (B) moxifloxacin analogs (MOX- N_i - M_j , MOX'- N_i - M_j), (C) ofloxacin analogs (OFL- M_j , OFL'- M_j)

analogues (8.510), 7-non-amino substituted analogues (10.731)), and ofloxacin analogues (7-amino substituted analogues (180), 7-non-amino substituted analogues (189)). The following designation scheme was used: ciprofloxacin analogues (7-amino substituted analogues (CIP- N_i - M_j), 7-non-amino

substituted analogues (CIP'- N_i - M_j)), moxifloxacin analogues (7-amino substituted analogues (MOX- N_i - M_j), 7-non-amino substituted analogues (MOX'- N_i - M_j)), and ofloxacin analogues (7-amino substituted analogues (OFL- M_j), 7-non-amino substituted analogues (OFL'- M_j)). Combining all subsets of analogues

Table 1 Fragments selection details for combinatorial enumeration. The building-blocks (R_7 -substructural fragments) with non-amino attachment point are signed with asterisk (*)

ID	Virtual combinatorial definition		Substructural fragments		Boolean output (Y/N)				ρ_{tot}
	R ₁	R ₇	R ₁	R ₇	R ₁		R ₇		
					Y (N_i)	N	Y (M_j)	N	
1.	R ₁ -NH ₂	R ₂ R ₃ -NH	126	363	116	10	106	257	12.296
2.	R ₁ -NH ₂	R ₂ -*	123	340	115	8	191	149	21.965
3.	R ₁ -NH ₂	R ₂ R ₃ -NH	123	347	115	8	74	273	8.510
4.	R ₁ -NH ₂	R ₂ -*	123	337	73	50	147	190	10.731
5.	N/A	R ₁ R ₂ -NH	N/A	348	N/A	N/A	180	168	180
6.	N/A	R ₁ -*	N/A	337	N/A	N/A	189	148	189

obtained by using the combinatorial algorithm, resulted in building a general virtual combinatorial library (*CombiTot*) with a total of 53,871 6-FQ structural analogs, which were subsequently used for assessing the drug-like properties (all structures are available as *.sdf file format in electronic supplementary material, online resource 1).

Drug-likeness assessment using chemometric approaches

Albeit this combinatorial library contains a considerable number of 6-FQ analogs (*CombiTot*, ρ_{tot} =53,871) it can be considered as a small sub-space in the available chemical space [30]. Since the combinatorial algorithm in first instance increased the molecular complexity, there is a low probability that each compound in such a virtual library will possess drug-like properties. Therefore, drug-likeness filtering was performed taking into consideration the rules of Lipinski [31] and Veber [32]. Using the ChemBioOffice pre-integrated cheminformatics functions [22], we developed a robust drug-likeness filtering tool integrating both rule-sets (Lipinski “rule of 5” and Veber rules) which was used for filtering of our general combinatorial library and defining the drug-like chemical sub-space of 6-FQ analogs. This filtering procedure yielded a list of 1,101 out of 53,871 virtual combinatorial 6-FQ analogs as promising compounds for further investigation. These compounds were further assessed for prediction of their unknown activity values ($pMIC_{pred-combi}$) using a pre-built QSAR model [21]. Combinatorially-generated 6-FQ analogs possessing drug-like properties (*CombiDL*, 1,101 analogs) are available as “electronic supplementary material” (*.sdf file format, online resource 2).

Prediction of the biological activity values using a derived non-linear neural networks (NN) model

Our previously published non-linear neural networks (NN) model (see [21] for details) was used for prediction of

biological activity values ($pMIC_{pred-combi}$) for the external combinatorial library of 1,101 novel unknown 6-FQ structural analogs. The model was built employing counter-propagation artificial neural networks (CP ANN) methodology using a dataset of 145 structurally-similar 6-fluoroquinolones (all analogs are available as *.sdf file format in electronic supplementary material, online resource 3) with experimentally-determined biological activity values ($pMIC_{exp}$) against *M. tuberculosis* and an extensive set of nearly 600 calculated 2D molecular descriptors. Heuristic algorithm and intercorrelation matrix were used for selection of statistically-significant molecular descriptors for activity. The Heuristic algorithm which is a suitable method for pre-selection of molecular descriptors is first calculating the one-parameter correlation equations between molecular descriptors and activity, eliminating all descriptors that do not fulfill the pre-defined criteria: (1) The Fisher *F*-test value for the one-parameter correlation with the descriptor is less than 1.00, (2) The squared correlation coefficient (R^2) for the one-parameter equation is less than R_{min}^2 (the default value is $R_{min}^2=0.1$), (3) *t*-test value is less than *t*1 (where R_{min}^2 and *t*1=1.5), and (4) the descriptor is highly intercorrelated with another descriptor (above r_{full} , where $r_{full}=0.99$), and this other descriptor has a higher squared correlation coefficient in the one-parameter equations based on these descriptors. With the remaining descriptors, the algorithm calculates all possible two- and more-parameter linear models [33]. Initially, several linear models with up to ten descriptors were developed. The frequency analysis of occurrence of the molecular descriptors between these models, resulted in selection of the most frequently occurring molecular descriptors which were used as input variables in neural-networks modeling part. Kohonen artificial neural networks (KANN) was employed for splitting the dataset into a training set (*Assay2*, 115 compounds) and an external validation set (*Assay2*, 30 compounds). The model was built on the training set using

CP ANN and internally validated employing the cross-validation leave-one-out procedure ($R_{tr}=0.96$, $R_{tr-cv}=0.62$, where R_{tr} designates the coefficient of correlation for the model, while R_{tr-cv} is coefficient of correlation for cross-validation leave-one-out) as well as externally validated for its predictive performances using the external validation set ($R_{val}=0.8454$, where R_{val} is coefficient of correlation for the external validation set). Selection of a subset of combinatorially-generated 6-FQ analogs for pharmacophore analysis was performed by defining a global hypothetical activity (GHA) range $0.00 \leq MIC_{pred-combi} [\mu g/mL] \leq 0.10$ based on the determined experimental data for the following 6-FQs: *Structure2* (levofloxacin, $MIC_{exp}=0.0115 \mu g/mL$), *Structure21* (clinafloxacin, $MIC_{exp}=0.01 \mu g/mL$), and *Structure121* (moxifloxacin, $MIC_{exp}=0.025 \mu g/mL$) [21]. This selection resulted in the extraction of 427 out of 1,101 6-FQ analogs which had their activity predicted inside the pre-defined GHA range. These analogs were used as a drug-like combinatorial library (*CombiLib*) of novel 6-FQs for three-dimensional pharmacophore analysis and molecular docking study (all structures are available as *.sdf file format in electronic supplementary material, online resource 4).

Ligand-based and structure-based pharmacophore modeling of the 6-fluoroquinolone analogs

Although both DNA gyrase A (GyrA) and B (GyrB) subunits have been solved by the x-ray crystallography [34, 35], currently a full experimental atomistic picture of the GyrA₂GyrB₂ tetramer in complex with the DNA and 6-fluoroquinolone molecules remains unidentified. On the other hand, recent seminal studies on the closely related type II topoisomerase – topoisomerase IV – the complexes formed by the *Streptococcus pneumoniae* ParC (equivalent of GyrA subunit) and ParE TOPRIM domains of the topoisomerase IV together with 6-fluoroquinolones intercalated in the gap between nucleotides of the DNA were solved [36]. These data provided the first solid atomistic insights into the mechanism of action of the fluoroquinolone antibacterial agents. As both type II topoisomerases (DNA gyrase and topoisomerase IV) share a close structural and functional resemblance we used both available crystal structures to perform pharmacophore modeling and molecular docking calculations. Furthermore, for the 6-FQ chemical class a good correlation between the measured in vitro IC₅₀ values and in vivo MIC activities was observed. This enables utilization of the in vivo MIC data also for the interpretation of the atomistic pharmacophore and docking calculations [37].

Pharmacophore modeling approach is one of the widely used concepts in modern drug discovery [34]. Several software tools available today are able to provide predictive

3D pharmacophore models in an automated fashion. LigandScout [18], a software tool for automatic pharmacophore model generation from the available structural information was employed for the visualization and exploration of the topoisomerase IV binding site in complex with known 6-FQ active agents (structure-based design approach) as well as for constructing 3D-pharmacophore models based only on the 6-FQ ligand structures (ligand-based design approach). Three ligand-topoIV-DNA cleavage complexes from *S. pneumoniae*, available from the Protein Data Bank (PDB) repository [pdb codes: 3FOE, 3FOF, 3K9F], were used for the 3D-pharmacophore models generation [36, 38]. The models were obtained by automatic recognition of the 6-FQ co-crystallized ligand structures (clinafloxacin (3FOE), moxifloxacin (3FOF), and levofloxacin (3K9F)) and the surrounding amino acid residues of the 6-FQ analogs binding site and by analysis of possible ligand-protein interactions. Subsequently, the 3D structure-based pharmacophore (SBP) models were generated automatically (assignment of the pharmacophoric features for each ligand separately and ligand alignment generation) together with excluded volume spheres.

Therefore, two structure-based pharmacophore (SBP) models: shared (SBP_{shared}, interpolation of the identified pharmacophore features) and merged (SBP_{merged}, unification of the identified pharmacophore features) were constructed. Furthermore, the experimental conformations of the three bound 6-FQ analogs, were used for building of ligand-based pharmacophore (LBP) model. For each of the molecules, 250 unique conformations were calculated and aligned to yield a ligand-based pharmacophore (LBP) model. Finally, both combinatorial (*CombiLib*) and experimental library (*Assay 2*) were converted into multi-conformer libraries (maximum 25 conformations for each molecule) and subsequently screened with built-in LigandScout pharmacophore VS engine to evaluate the matching of the investigated compounds to the derived set of pharmacophore features.

Molecular docking of the 6-fluoroquinolone analogs

Molecular docking of the 6-fluoroquinolone molecules into the type II topoisomerase crystal structure

The docking experiments were performed by using GOLD docking engine [39], and protein structure with the PDB code 3K9F was used to define the binding site located around the experimental coordinates of the bound 6-FQ inhibitor levofloxacin resulting in a cavity radius of 12.5 Å. Each investigated molecule was docked 10 times into the binding site by applying the following parameters of the GOLD genetic algorithm (GA) (population size=100,

selection pressure=1.1, no. of operations=100000, no. of islands=5, niche size=2, migrate=10, mutate=95, cross-over=95). Early termination was allowed if the top three solutions were within 1.5 Å of the root-mean-squared-deviation (RMSD) value. For the assessment of the binding affinity, GOLDScore scoring function was used [39].

The quality of the generated binding poses were validated by re-docking the levofloxacin into its binding site and exploring the positioning of the 145 6-FQ molecules with the available experimental MIC data [40]. Finally, all compounds of the generated reduced combinatorial library (*CombiLib*, 427 compounds, online resource 4), were docked into the defined binding site using the same procedure.

Results and discussion

Virtual combinatorial chemistry approach as a strategy for generating chemical libraries of structurally-similar analogs for a given compound of interest as well as their investigation implementing highly-sophisticated cheminformatics algorithms are extensively documented [41–45]. There are two crucial steps which must be taken into account for combinatorial generation of structural analogs for the entity under investigation: the synthetic procedure (generic synthetic reaction) for preparation of the compound as well as the introduction of substituents onto a scaffold [26]. In our case, a focused virtual combinatorial library of novel 6-FQ analogs (*CombiTot*) utilizing the established combinatorial algorithm for SAR-based scaffold permutation was obtained.

Druggability properties assessment and defining the drug-like chemical sub-space

The drug-likeness assessment of the combinatorially-generated library of 6-FQ structural analogs (*CombiTot*, 53.871 compounds) was carried out by calculating the druggability properties: MW, AlogP (Ghose-Crippen's atom-based model for logP, i.e., partition coefficient for *n*-octanol/water bi-phase system), *n*HBD, *n*HBA, MP_{SA} (molecular polar surface area), and *n*RB.

The calculated druggability properties were analyzed using the property distribution comparison methodology (histogram-type of analysis, Table 2, Fig. 4) [46] between the dataset of known 6-FQs used in the development of the NN model (*Assay2*, 145 compounds, Fig. 4A) [21] and the combinatorial (*CombiTot*, 53.871 compounds, Fig. 4B).

The results of the statistical analysis show that only one of the calculated properties for the experimental compounds (Fig. 4A) follows normal Gaussian distribution (*n*HBD_{exp}), whereas the rest of the calculated properties (MW_{exp}, MP_{SA}_{exp}, AlogP_{exp}, *n*HBA_{exp}, and *n*RB_{exp}) are asymmetrically distributed. The peak-analysis (the top-point on the

Gaussian curve where the distribution of the calculated property reaches 50%) showed acceptable values for drug-likeness: MW_{exp}=468, AlogP_{exp}=−0.07, *n*HBD_{exp}=2, *n*HBA_{exp}=8, MP_{SA}_{exp}=103, and *n*RB_{exp}=5. The corresponding mid-50% values (the interval between 25% and 75% of the distribution, i.e., first (Q1) and third (Q3) calculated quartile, respectively) were obtained employing the quartile calculation: MW_{exp-mid50%}=382–566, AlogP_{exp-mid50%}=−(0.848)–(+0.394), *n*HBD_{exp-mid50%}=2, *n*HBA_{exp-mid50%}=6–9, MP_{SA}_{exp-mid50%}=74.38–124.77, and *n*RB_{exp-mid50%}=3–6 (Table 2A). These results are in accordance with the Lipinski and Veber rule-sets, respectively, and clearly define the domain in which the compounds possess drug-like characteristics [31, 32].

On the other hand, an obvious difference in the property distribution was observed for the compounds within the virtual combinatorial set (Fig. 4B). A normal Gaussian distribution was observed for five calculated properties (MW_{combi}, AlogP_{combi}, *n*HBA_{combi}, MP_{SA}_{combi} and *n*RB_{combi}), while the *n*HBD_{combi} parameter follows an asymmetric distribution. The peak-analysis for the combinatorial set resulted in following values: MW_{combi}=621, AlogP_{combi}=4.87, *n*HBD_{combi}=2, *n*HBA_{combi}=9, MP_{SA}_{combi}=157, and *n*RB_{combi}=7, whereas the corresponding mid-50% values were in the following boundaries: MW_{combi-mid50%}=578–667, AlogP_{combi-mid50%}=3.976–5.911, *n*HBD_{combi-mid50%}=1–2, *n*HBA_{combi-mid50%}=8–10, MP_{SA}_{combi-mid50%}=137.36–174.89, and *n*RB_{combi-mid50%}=6–8 (Table 2B).

The increased values (MW_{combi} and MP_{SA}_{combi}) pointed toward the increased molecular complexity [47] and a decreased probability for a good ligand-protein interaction [48]. Thus, a pre-filtering of the combinatorial library was performed by implementing a comprehensive Boolean-type (T/F (true/false)) drug-likeness filtering algorithm (*CombiVL*; MW<500, AlogP<5.0, *n*HBD≤5, *n*HBA≤10, MP_{SA}≤140, *n*RB≤10) integrating both rule-sets (Lipinski “rule-of-five” and Veber rules) [31, 32].

Such a filtering procedure resulted in eliminating all 6-FQ analogs which do not satisfy the above criteria (in the *CombiTot* library 52.770 eliminated compounds were marked as false (F)). The retained 6-FQ structural analogs (total 1.101 compounds) define the drug-like chemical sub-space (*CombiDL*, online resource 2) which was subsequently used for prediction of the biological activity values (*p*MIC_{pred-combi}) employing our derived NN model [21].

Prediction of the biological activity values for the novel combinatorially-generated 6-fluoroquinolone drug-like analogs and activity-based subset selection

A derived seven parameter neural-networks (NN) model (*Assay2*, 145 compounds, online resource 3) [21] was used for prediction of the biological activity values (*p*MIC_{pred-combi})

Table 2 Property distribution analysis of the experimental and the combinatorial set, respectively (No., number of compounds in the dataset; Prop., calculated property; Mean, the mean value; Min, minimum value; Q1, first quartile; Median, the median value; Q3,third quartile; Max, the maximum value). (A) the experimental set (145 compounds), (B) the combinatorial set (*CombiTot*, 53.871 compounds)

A)	No.	Prop.	Mean	Min	Q1	Median	Q3	Max
Experimental set	145	MW _{exp}	467.968	275.280	382.380	419.420	566.490	801.240
		AlogP _{exp}	−0.069	−2.453	−0.848	−0.337	0.394	6.251
		nHBD _{exp}	1.952	1	2	2	2	3
		nHBA _{exp}	7.621	4	6	7	9	14
		MPSA _{exp}	102.983	57.610	74.380	86.150	124.770	186.310
		nRB _{exp}	4.959	1	3	5	6	11
B)	No.	Prop.	Mean	Min	Q1	Median	Q3	Max
Combinatorial set	53.871	MW _{combi}	621.099	329.280	578.410	623.540	666.620	814.440
		AlogP _{combi}	4.872	−3.735	3.976	5.002	5.911	9.265
		nHBD _{combi}	1.958	1	1	2	2	6
		nHBA _{combi}	8.868	4	8	9	10	13
		MPSA _{combi}	156.742	65.780	137.360	155.030	174.890	272.820
		nRB _{combi}	6.807	2	6	7	8	12

for the previously filtered combinatorially-generated drug-like 6-FQ structural analogs (*CombiDL*, 1.101 compounds, online resource 2). Using this developed model the biological activity ($pMIC_{pred-combi}$) in the series of novel unknown 6-FQ analogs is correlated to a set of seven constitutional, topological, and electrostatic parameters (Table 3).

As presented in Table 3, nR09 belongs to the class of pure constitutional parameters, GATS8v, YZS/YZR, X1A, and PW3, are molecular descriptors which belong to the class of topological parameters, while the parameters JGI2 and JGI3, belong to the class of pure electrostatic parameters. These molecular descriptors, which in general accentuate the importance of molecular shape can be linked to the accommodation of the main 6-FQ scaffold within the GyrA subunit binding pocket. Since the QSAR model was built employing a series of structurally-similar 6-FQ analogs with experimentally determined biological activity values, one would expect similar biological response of these compounds within the same protein target. In this respect, we believe that GATS8v parameter alone, as molecular descriptor describing the importance of the atomic van der Waals volumes of our 6-FQs, is of particular significance for the biological activity, mainly through establishing proper steric complementarity between the ligand and the enzyme. On the other hand, the X1A parameter is a typical topological molecular descriptor, i.e., a pharmacophore fingerprint that carries the connectivity information of 6-FQ analogs, probably through establishing π - π stacking interactions between the planar aromatic/heteroaromatic systems in the 6-FQs and the GyrA/DNA, respectively.

The electrostatic descriptors JGI2 and JGI3 suggest that anti-mycobacterial activity is potentially dependent on the charge indices for the oxygen of the carboxyl and carbonyl

group within the main core. They also describe the possibility for establishing hydrogen-bonding interactions between these substituents and the amino acid residues within the GyrA binding pocket. Moreover, these parameters suggest the possibility of establishing an electrostatic interaction between the F atom at position 6 and the target protein which may result in an enhanced binding of the 6-FQ analogs to the complex.

Activity-based subset selection for molecular modeling calculations

The predicted biological activity values ($pMIC_{pred-combi}$) for the combinatorially-generated drug-like 6-FQ analogs are in the range between $0.0125 < pMIC_{pred-combi} < 0.9174$, while the corresponding $MIC_{pred-combi}$ values, obtained after $pMIC_{pred-combi}$ de-normalization and anti-logarithmization, are in the range between $0.0021 < MIC_{pred-combi} [\mu g/mL] < 6.3726$. Using a GHA range described previously, a subset of 427 6-FQ analogs (*CombiLib*, online resource 4) was extracted from the pool of total 1.101 compounds (*CombiDL*). Such a selection procedure ensures that each 6-FQ analog in the MIC-based isolated subset (*CombiLib*) possess biological activity ($0.4809 < pMIC_{pred-combi} < 0.9174$; $0.0021 < MIC_{pred-combi} [\mu g/mL] < 0.1000$) against *M. tuberculosis*. The selected combinatorial subset (*CombiLib*, 427 compounds), was subsequently used as an external library in the three-dimensional pharmacophore analysis and molecular docking study.

6-FQs pharmacophore modeling study

Using LigandScout's the integrated automatic pharmacophore generation algorithm, three pharmacophore models

Fig. 4 Histogram-type of analysis for druggability properties assessment (property distribution). (A) the experimental set (*Assay2*, 145 compounds), (B) the combinatorial set (*CombiTot*, 53,871 com-

pounds). The property distribution is fitted with normal distribution to the histogram of data

(LBP, SBP_{shared} , and SPB_{merged}) were constructed as described previously. Since the automatic pharmacophore generation yielded all possible pharmacophoric features, existing knowledge about the SAR of 6-FQs [16] was employed for pharmacophoric simplification of all models. Such a simplification approach resulted in a total of five pharmacophoric features per model (one aromatic ring, two hydrophobic features, one hydrogen bond

donor, and one negative ionizable area). The obtained three-dimensional pharmacophore models (LBP, SBP_{shared} , and SPB_{merged}), served as highly effective in silico filtering tools for subsequent VS of the 6-FQ ligands (Fig. 5).

Since the core idea of the three-dimensional pharmacophoric concept is the selection of active compounds among a pool of inactive molecules [18], our generated

3D pharmacophore models (LBP, SBP_{shared}, and SPB_{merged}) were validated for their recognition performances as in silico filters in a VS experiment using a dataset of known 6-FQs with experimentally-measured biological activity values (*Assay2*, 145 compounds) [21]. Initially, the LBP model identified 58 active out of total 145 compounds, while the two SBP models (SBP_{shared} and SPB_{merged}) identified 62 and 49 compounds, as active compounds respectively. The experimentally-determined biological activity values for the successfully filtered compounds are in the range between $0.001 < \text{MIC}_{\text{exp}} [\mu\text{g}/\text{mL}] < 3.500$, of which around 90% were determined as highly-active compounds with biological activity values in the range between $0.001 < \text{MIC}_{\text{exp}} [\mu\text{g}/\text{mL}] < 0.900$. The 6-FQs obtained by the pharmacophore-based VS procedure (LBP (58), SBP_{shared} (62), and SPB_{merged} (49)), were visually inspected for molecular fitness within each of the pre-defined pharmacophoric features of the models (one aromatic ring, two hydrophobic features, one hydrogen bond donor, and one negative ionizable area).

In order to assess the visual inspection more precisely, a Boolean-type of signing (T/F (true/false)) was introduced. Each visually-determined match between the 6-FQ's substructural elements and the pharmacophore-model features was marked as true (T). In addition, the biological activity values (MIC_{exp}) of the investigated compounds were implemented as a feature in the evaluation process using the GHA range. Thus, the compounds with biological activity values in the GHA range were marked as true (T), while the rest of the compounds were marked as false (F). Therefore, only the compounds with (T) outcome for all the pre-defined pharmacophoric-model features as well as lying within the GHA range (electronic supplementary material, online resource 5, experimental set), can be marked as highly favorable 6-FQs (LBP (20 highly active 6-FQs out of 58), SBP_{shared} (19 highly active 6-FQs out of 62), and SPB_{merged} (8 highly active 6-FQs out of 49). Online resource 5, shows that selected compounds from the experimental set (*Structure24*, *Structure39*, *Structure75*, and *Structure110*), which are all potent compounds, were identified as active compounds by all three pharmacophore models.

The structural analysis of the selected compounds (online resource 5, see “experimental set” sheet), shows that the cyclopropyl group is the most frequently used functional group at position 1 of the main 6-FQ scaffold, whereas position 7 can be successfully substituted by a range of substructural fragments, mainly heterocyclic systems of which 5- and 6-membered N-hetero systems (aminopyrrolidines, piperazines), are the most optimal for anti-mycobacterial activity. These results suggest the SAR

rules for optimal anti-mycobacterial activity of the 6-FQs [16] and that the three-dimensional pharmacophore concept can be successfully employed as a highly-effective in silico filtering tool [19].

Following the validation on the experimental set [21], the same 3D pharmacophore models were used for assessing the combinatorially-generated subset (*CombiLib*, 427 novel compounds). The LBP model initially identified 95 active out of a total of 427 compounds as hits, whereas the two structure-based pharmacophore models (SBP_{shared} and SBP_{merged}) identified 95 and 77 compounds, respectively. Since the combinatorial subset was built defining a GHA range, all of the 6-FQ analogs in such a library are hypothetically active against *M. tuberculosis*, regarding the $\text{MIC}_{\text{pred-combi}}$ values ($0.0021 < \text{MIC}_{\text{pred-combi}} [\mu\text{g}/\text{mL}] < 0.1000$). Therefore, a visual determination of the matches using (T/F) designation between the structural elements of the novel actively-recognized 6-FQs hits (LBP (95), SBP_{shared} (95), and SBP_{merged} (77)) and pharmacophoric features within the models was implemented to identify the most optimal structural features.

The selected compounds (LBP (32 out of 95), SBP_{shared} (26 out of 95), and SBP_{merged} (31 out of 77)), belong to three general classess of combinatorially-generated 6-FQ compounds: ciprofloxacin analogs (7-amino (CIP- N_i - M_j)/7-nonamino derivatives (CIP'- N_i - M_j)), moxifloxacin analogs (7-nonamino derivatives (MOX'- N_i - M_j)) and ofloxacin analogs (7-amino (OFL- M_j)/7-nonamino derivatives (OFL'- M_j)).

The frequency analysis of occurrence of the substructural fragments (throughout the models output,

Table 3 The most important 2D molecular descriptors (pharmacophore fingerprints) for the activity (T, topological; C, constitutional; E, electrostatic)

ID	Descriptor	Source	Definition	Class
1.	GATS8v	DRAGON	Geary autocorrelation-lag8/weighted by atomic van der Waals volumes	T
2.	nR09	DRAGON	Number of 9-membered rings	C
3.	JGI2	DRAGON	Mean topological charge index of order 2	E/T
4.	JGI3	DRAGON	Mean topological charge index of order 3	E/T
5.	YZS/YZR	CODESSA	YZ Shadow/YZ Rectangle	T
6.	X1A	DRAGON	Average connectivity index chi-1	T
7.	PW3	DRAGON	Path/Walk 3-Randic shape index	T

Fig. 5 The three pharmacophore models obtained after pharmacophoric features reduction (a total of five features per model). (A) ligand-based pharmacophore (LBP) model, (B) structure-based shared pharmacophore (SBP_{shared}) model, (C) structure-based merged pharmacophore (SBP_{merged}) model, (D) The most promising six combinatorial compounds obtained after pharmacophore models VS campaign and aligned into the SBP_{shared} pharmacophore model

electronic supplementary material, online resource 5, see “combinatorial set” sheet) attached at positions 1 and 7 of the main 6-FQ core - explicitly shows that the most frequently appearing fragment at position 1 (18 times) is the building-block benzo[d]oxazole marked as $N_i=028$ in the CIP'-analogs and $N_i=016$ in the MOX'-analogs, respectively, while the most frequently appearing fragments at position 7 of the main scaffold are the building-blocks: 1*H*-pyrazolo[3,4-*b*]pyridine (16 times) marked as $M_j=148$ in the CIP'-analogs, $M_j=116$ in the MOX'-analogs, and $M_j=147$ in the OFL'-analogs, respectively, and 1*H*-pyrazol-5(4*H*)-one (15 times) marked as $M_j=186$ in the CIP'-analogs and $M_j=144$ in the MOX'-analogs. In summary, six compounds were selected (CIP'-028-148, CIP'-028-186, MOX'-016-116, MOX'-016-144, OFL'-147, and OFL-148) as a result of pharmacophore modeling assessment (Table 4).

Interestingly, these compounds (except for OFL-148, i.e., an ofloxacin analog) belong to the 6-FQ analogs structurally different from ciprofloxacin and moxifloxacin with non-amino substructural fragments at position 7. The predicted biological activity values for these 6-FQ analogs are in the range $0.0035 < \text{MIC}_{\text{pred-combi}} [\mu\text{g/mL}] < 0.1000$. The selected combinatorially-generated 6-FQ analogs, were subsequently used for assessing the possible interactions with experimentally-bound 6-FQ protein structure, as an integrated part of the

external combinatorial subset (*CombiLib*, 427 novel compounds) in the molecular docking study.

Molecular docking study

The molecular docking study on both, the experimental set (*Assay2*, 145 compounds) with experimentally-determined biological activity values and the combinatorial one (*CombiLib*, 427 compounds), was performed by using the experimentally-determined 6-FQs binding site of the type II topoisomerase protein (3K9F). In a previously published docking study, only GyrA protein subunit without full complex details (DNA and GyrB position), was used to assess the possible binding modes [49]. The co-crystallized 6-FQ inhibitor levofloxacin [38] was used for comparison as well as to analyze the geometric and structural properties of the docked 6-FQ compounds in the protein. The re-docking validation procedure [40] of levofloxacin into its binding site was successful as GOLD was able to reproduce the experimental bound conformation with high accuracy.

The post-docking VS analysis was divided into two levels. At the first level, the geometric properties of both sets of compounds were assessed by visually inspecting each 6-FQ dock position relative to the experimental conformation of levofloxacin [38]. The following geometric properties were compared: the visual

Table 4 The combinatorial compounds selected according to the frequency analysis of the sub-structural fragments (throughout the pharmacophore models output) attached at position R₁ and R₇ of the main 6-FQ scaffold, respectively, together with their corresponding

pharmacophore fitness score (PFS). The selected compounds with highest predicted biological activity values ($MIC_{pred-combi}$), are highlighted in gray

ID	Chemical structure	R ₁	R ₇	$MIC_{pred-combi}$	$pMIC_{pred-combi}$	PFS
1.	 CIP'-028-148	028	148	0.0499	1.3022	54.39
2.	 CIP'-028-186	028	186	0.1000	1.0002	57.20
3.	 MOX'-016-116	016	116	0.0499	1.3022	57.21
4.	 MOX'-016-144	016	144	0.1000	1.0002	57.28
5.	 OFL'-147	N/A	147	0.0499	1.3022	57.15
6.	 OFL-148	N/A	148	0.0035	2.4580	57.06

orientation (how the screened 6-FQ compound is oriented relative to the levofloxacin position), visual fitness (how well the screened 6-FQ compound fits the experimental

levofloxacin conformation), and the number of the matching pharmacophoric features (how many common pharmacophoric features, generated for each docked

conformation, are shared by both screened 6-FQ compound and levofloxacin). Similarly to the pharmacophore analysis, a Boolean-type of signing (T/F (true/false)) was employed for assessing the visual orientation and visual fitness parameters, while the matching pharmacophoric features were defined numerically. The screened compounds which share 4–5 pharmacophoric features present in bound levofloxacin, were signed as true (T). Therefore, the result of the first level assessment can be defined as a sum of the obtained Boolean answers for all three investigated properties (electronic supplementary materials, online resource 6).

First level post-docking analysis of the experimental set of 6-FQ analogs docked into the 3K9F binding pocket (online resource 6, see “Exp-3K9F” sheet) resulted in 102 (T) marked compounds out of 145, of which 38 compounds have measured biological activity values within $0.001 < \text{MIC}_{\text{exp}} [\mu\text{g/mL}] < 0.1$. Moreover, as shown in Fig. 6A, a favorable positioning of these 6-FQ compounds into the 3K9F binding pocket could be observed. The structural analysis of these 38 compounds, once again showed that the cyclopropyl group is the most frequently appearing substituent at position 1 of the main 6-FQ scaffold (33 times), while the piperazinyl group attached at position 7 appeared 24 times. These results are again in accordance with the previous experimental SAR findings (online resource 6, “experimental set” sheet) [16].

The first level post-docking analysis of the 6-FQ analogs in the combinatorial set (*CombiLib*, 427 compounds) docked into the 3K9F binding pocket (electronic supplementary materials, online resource 6, see “Combi-3K9F” sheet) resulted in 166 (T) marked compounds out of 427.

Interestingly, comparing with the previous assessment using pharmacophore models filters where none of the MOX- N_i - M_j analogs was recognized as active for further investigation, the post-docking Boolean procedure performed here identified only one moxifloxacin analog (MOX-028-064) as geometrically suitable 6-FQ compound in 3K9F binding pocket. The 6-FQ combinatorial hit analogs belonging to the other five structural classes (CIP- N_i - M_j , CIP'- N_i - M_j , MOX'- N_i - M_j , OFL- M_j , OFL'- M_j) were successfully identified. According to the combinatorial 6-FQ (T) outcome obtained, 166 compounds from the combinatorial set have geometric properties, comparable to the co-crystallized levofloxacin (Fig. 6B).

The frequency analysis of occurrence of the substructural fragments attached at positions 1 and 7 of these 166 compounds, indicated that the most frequently appearing fragments at position 1 are the building-blocks: benzo[*d*]oxazole (46 times) marked as $N_i=028$ in the CIP'-analogs and in the CIP-analogs, and $N_i=016$ in the MOX'-analogs, respectively, and 1-(pyridin-3-yl)ethanone (11 times) marked as $N_i=102$. The most frequently appearing frag-

Fig. 6 Results of docking into the 3K9F protein binding pocket. (A) 38 compounds from the experimental set with acceptable geometric properties. (B) 166 compounds from the combinatorial set with

acceptable geometric properties. Crucial amino acid residues from the 3K9F 6-FQs binding site are displayed [35]

ments at position 7 of the main scaffold are the building-blocks: 4*H*-furo[3,2-*c*]pyran-4-one (8 times) marked as $M_j=006$, 1*H*-pyrazolo[3,4-*b*]pyridine (7 times) marked as $M_j=148$, and 1*H*-pyrazole (7 times) marked as $M_j=176$.

Except compound OFL-148, all selected 6-FQs from the performed pharmacophore analysis (CIP'-028-148, CIP'-028-186, MOX'-016-116, MOX'-016-144, OFL'-147) were successfully identified as compounds with favorable three-dimensional binding geometry as well (online resource 6, see “Combi-3K9F” sheet). Therefore, all of the selected 6-FQ analogs from the experimental set (3K9F (102)) and the combinatorial one (3K9F (166)), respectively, were subsequently used for further investigation at the second level post-docking analysis.

At the second level of the post-docking analysis, the intermolecular interaction properties of the selected geometrically suitable 6-FQs from both experimental and combinatorial sets were investigated (electronic supplementary materials, online resource 8). The crystal structure of the *S. pneumoniae* topoisomerase IV protein in complex with the co-crystallized ligand levofloxacin (3K9F) was used as a starting point for measuring the interaction distances between the ligand's functional groups and the surrounding amino acid residues [38]. A set of five key interactions between the levofloxacin and the protein were used as standard measures: (R_3 -COO⁻)-(HO-Ser79) [$d_{3K9F-ref-1}=3.18$ Å], (R_3 -COO⁻)-(HO-Ser79) [$d_{3K9F-ref-2}=3.35$ Å], (R_3 -COO⁻)-(HO-Arg117) [$d_{3K9F-ref-3}=3.01$ Å], ($R_7=N-CH_3$)-(H₂N⁺=Arg456) [$d_{3K9F-ref-4}=3.30$ Å], and ($R_7=N-CH_3$)-(OOC-Glu475) [$d_{3K9F-ref-5}=4.38$ Å]. Since the biological systems are not static and are characterized with dynamic features, a distance tolerance of ± 1 Ångstrom unit around each of the standard interatomic distance values was employed: (R_3 -COO⁻)-(HO-Ser79) [$d_{3K9F-1}=2.18$ -4.18 Å], (R_3 -COO⁻)-(HO-Ser79) [$d_{3K9F-2}=2.35$ -4.35 Å], (R_3 -COO⁻)-(HO-Arg117) [$d_{3K9F-3}=2.01$ -4.01 Å], ($R_7=N-CH_3$)-(H₂N⁺=Arg456) [$d_{3K9F-4}=2.30$ -4.30 Å], and ($R_7=N-CH_3$)-(OOC-Glu475) [$d_{3K9F-5}=3.38$ -5.38 Å].

The second level post-docking analysis in 3K9F (online resource 7, see “Exp-3K9F” sheet) selected 45 out of 102 compounds from the experimental set as optimal 6-FQ compounds (regarding the interatomic distances). The biological activity values for these compounds are in the range between $0.001 < MIC_{exp} [\mu g/mL] < 6.700$ of which approximately 38% have good measured biological activity ($0.001 < MIC_{exp} [\mu g/mL] < 0.100$). The structural analysis once again showed that the most frequently occurring substituent at position 1 of the main 6-FQ scaffold is the cyclopropyl group (15 times), whereas the most frequent substituent attached at position 7 of the hit molecules is the piperazinyl group (12 times), whose results are in accordance with the established 6-FQs SAR rules (Fig. 1) [16].

The second level post-docking analysis of the combinatorial compounds resulted in selection of a total of 11 out of 166 compounds (online resource 7, see “Combi-3K9F” sheet). These compounds mainly belong to the class of 7-nonamino substituted ciprofloxacin analogs (CIP'- N_i - M_j (nine compounds), one 7-amino substituted ciprofloxacin analog (CIP- N_i - M_j), and one 7-amino substituted ofloxacin analog (OFL- M_j). No moxifloxacin analogs (MOX- N_i - M_j and MOX'- N_i - M_j) and 7-nonamino substituted ofloxacin analogs (OFL'- M_j) were found that could be fitted in this set of defined interatomic distance boundaries [38].

The frequency analysis of occurrence of the substituents attached at position 1 and 7 of the main scaffold, once again pinpointed the fragment marked as $N_i=028$ as the most frequently appearing substituent at position 1 (5 times), while position 7 can be successfully substituted with a range of different groups. Incorporation of these fragments thus forms novel 6-FQ compounds that could also serve as novel target compounds in the synthetically-driven lead optimization. The list of compounds that complements the previous selection in Table 4 is presented in Table 5.

Conclusions

In the present study a variety of chemometric and molecular modeling approaches were integrated into a powerful complex scheme capable of constructing as well as evaluating a virtual combinatorial library of 6-fluoroquinolone analogs (6-FQs). This 6-FQs library was designed by employing the synthetically-driven ligand generation rules feasible of yielding ligands which can be readily synthesized and have a predicted inhibitory activity toward GyrA.

The results can be summarized as follows: (1) a large number of virtual 6-fluoroquinolone analogs (53,871 compounds) were generated by a combinatorial generation of all substituted amine and non-amine compounds at positions 1 and 7, respectively. The selection of a drug-like set of 427 compounds from the library within the GHA range was performed by using drug-likeness filters (a combined Lipinski-Veber filtering tool based on the Lipinski's and Veber's rule-sets for drug-likeness) and our previously developed and validated neural-networks (NN) chemometric model (built on a dataset of structurally-similar 6-FQ compounds with experimentally-determined biological activity values by employing a combined QSAR modeling strategy, i.e., linear modeling as well as non-linear modeling using Kohonen and counter-propagation artificial neural networks) [21]. (2) Experimental data on the structurally-similar topoisomerase-IV enzyme in complex with levofloxacin was used to construct structure-based as well as ligand-based pharmacophore models to evaluate the most promising 6-FQs obtained by chemometric methods. The

Table 5 The combinatorial compounds extracted as most suitable after the second level VS analysis into the 3K9F binding pocket, together with their GoldScore Fitness (GSF) scoring function and the corresponding inter-atomic distances (in Ångstrom units) between the

6-FQ sub-structural fragments and the crucial amino acid residues important for the biological activity. The compounds with highest predicted biological activity values ($MIC_{pred-combi}$), are highlighted in gray

ID	Chemical structure	MIC <i>pred-combi</i>	pMIC <i>pred-combi</i>	GSF	d _{3K9F-1} [2.18-4.18]	d _{3K9F-2} [2.35-4.35]	d _{3K9F-3} [2.01-4.01]	d _{3K9F-4} [2.30-4.30]	d _{3K9F-5} [3.38-5.38]
1.	 CIP'-028-059	0.0499	1.3022	78.29	3.31	3.92	3.67	3.99	5.07
2.	 CIP'-028-065	0.1000	1.0002	87.09	2.88	3.27	3.29	4.25	4.81
3.	 CIP'-028-125	0.0021	2.6818	76.16	3.49	3.86	3.24	3.40	5.15
4.	 CIP'-028-154	0.0021	2.6818	80.90	3.16	3.51	2.80	4.19	5.37
5.	 CIP'-028-156	0.0783	1.1065	72.18	3.45	4.31	2.62	2.64	5.00

models were also validated using available experimental 6-FQs data from the literature. (3) Finally, these compounds were docked into the topo-IV binding pocket and the interactions of 6-FQ analogs from virtual library as well as

experimental database with the surrounding amino acid residues were compared and analyzed.

The outcome of this study shows that promising compounds for further investigation originate from all three

Table 5 (continued)

6.		0.0130	1.8851	72.26	3.17	3.51	3.31	3.92	3.43
	CIP'-037-006								
7.		0.1000	1.0002	71.96	2.96	3.84	3.92	4.08	3.96
	CIP'-087-073								
8.		0.0401	1.3970	71.79	3.09	3.51	3.60	3.58	5.33
	CIP'-095-186								
9.		0.1000	1.0002	83.74	3.94	4.33	2.89	4.12	4.91
	CIP'-102-113								
10.		0.0681	1.1666	74.36	3.71	4.17	2.83	4.29	5.29
	CIP-103-102								
11.		0.0130	1.8851	72.53	3.34	3.65	3.00	3.01	3.73
	OFL-127								

chemical classes (ciprofloxacin, moxifloxacin, and ofloxacin analogs), whereas the ciprofloxacin chemical class

yielded the highest number of hits (CIP'-028-059, CIP'-028-125, CIP'-028-148, CIP'-028-154, CIP'-028-156, CIP'-

037-006, CIP'-095-186, and CIP-103-102). Furthermore, the detailed analysis of occurrence of the substructural fragments present at positions R₁ and R₇ of the 6-FQ hit molecules revealed several novel attractive fragments, such as for R₁: [(N_i=028, benzo[d]oxazole), (N_i=037, 2-(hydroxymethyl)phenol), (N_i=095, 1-methoxy-2-methylbenzene), and (N_i=103, 1-(pyridin-3-yl)ethanone)] and for R₇: [(M_j=006, 4H-furo[3,2-c]pyran-4-one), (M_j=059, 8-chloro-[1, 2, 4]triazolo[4,3-b]pyridazine), (M_j=102, 3-methyl-1H-pyrazol-5(4H)-one), (M_j=125, 3-(2-methyl-1,3-dioxolan-2-yl)aniline), (M_j=148, 1H-pyrazolo[3,4-b]pyridine), (M_j=154, methyl 5,6-dihydro-4H-cyclopenta[b]thiophene-2-carboxylate), (M_j=156, 1-(2-aminopyridin-3-yl)ethanone), and (M_j=186, 1H-pyrazol-5(4H)-one)], respectively, that satisfied the screening conditions at all levels. In conclusion, we hope that the results of our mixed chemometric-molecular modeling study will assist in providing new SAR guidelines to the lead optimization stage of the 6-FQ's drug design and thus enable the design of the novel - much-needed - antibacterial agents.

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