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The iterative application of a large chemical space in the drug discovery process

Abstract

Aim. To demonstrate the advantages of large-scale virtual libraries generated using chemical protocols previously validated in primary steps of the drug discovery process.

Results and discussion. Two validated parallel chemistry protocols reported earlier were used to create the chemical space. It was then sampled based on diversity metric, and the sample was subjected to the virtual screening on BRD4 target. Hits of virtual screening were synthesized and tested in the thermal shift assay.

Experimental part. The chemical space was generated using commercially available building blocks and synthetic protocols suitable for parallel chemistry and previously reported. After narrowing it down, using MedChem filters, the resulting sub-space was clustered based on diversity metrics. Centroids of the clusters were put to the virtual screening against the BRD4 active center. 29 Hits from the docking were synthesized and subjected to the thermal shift assay with BRD4, and 2 compounds showed noticeable dTm.

Conclusions. A combination of cheminformatics and molecular docking was applied to find novel potential binders for BRD4 from a large chemical space. The selected set of predicted molecules was synthesized with a 72 % success rate and tested in a thermal shift assay to reveal a 6 % hit rate. The selection can be performed iteratively to fast support of the drug discovery.

Keywords: chemical space; 1,2,4-triazole; tetrazole; BRD4; thermal shift assay

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Ітеративне використання великих хімічних просторів у пошуку лікарських засобів

Анотація

Мета. Продемонструвати переваги віртуальних бібліотек великого розміру, згенерованих за валідованими раніше хімічними протоколами, на перших етапах пошуку лікарських засобів.

Результати та їх обговорення. На основі двох валідованих методів синтезу, придатних для паралельної хімії, описаних раніше, було створено хімічний простір. На основі різноманітності з одержаної віртуальної бібліотеки зроблено вибірку, яку було піддано віртуальному скринінгу щодо активного центру білка BRD4. Хіти віртуального скринінгу було синтезовано й перевірено за допомогою диференційної сканувальної калориметрії.

Експериментальна частина. На основі комерційно доступних вихідних реагентів та раніше репрезентованих синтетичних протоколів, придатних для паралельної хімії, згенеровано хімічний простір. Простір було зменшено за рахунок застосування медхімічних фільтрів; результатний підпростір було кластеризовано за критерієм різноманітності. Центроїди кластерів було піддано молекулярному докінгу щодо активного центру білка BRD4. За результатами проведеного докінгу, синтезовано 29 хітів, які було піддано диференційній сканувальній калориметрії з білком BRD4; з цим 2 сполуки продемонстрували помітний зсув точки топлення.

Висновки. Для пошуку нових потенційних лігандів BRD4 у великому хімічному просторі було застосовано комбінацію хемоінформатики і молекулярного докінгу. Набір молекул, що мали високу передбачену активність, було синтезовано з успішністю 72%. Серед синтезованих сполук виявлено первинні хіти (6% сполук). Подібний процес можна повторювати ітеративно для швидкої підтримки розроблення ліків.

Ключові слова: хімічний простір; 1,2,4-триазол; тетразол; BRD4; диференційна сканувальна калориметрія

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Supporting information: Analytical data for the compounds synthesized. Physicochemical properties of the generated chemical space. Results of TSA. The SI file is freely available at http://ophcj.nuph.edu.ua.

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■ Introduction

A high throughput screening (HTS) has been a tool for medicinal chemists for decades [1, 2]. These days, however, direct use of HTS at the first stages of the drug discovery rarely happens. The drawbacks of this method are its cost and limited range of compounds available in stock – and no solid guarantees are given that the experiment yields potent compounds.

To substantially decrease the required experimental costs, the virtual screening, including molecular modeling, molecular docking, and AI/ML-based (Artificial Intelligence/Machine learning) approaches have been widely introduced and successfully utilized [3–5]. The latter allows screening in-stock and virtual compounds resulting in molecules with favorable profiles, which can be evaluated before involving the experimental part. Many approaches exist to generate a virtual library [6]. But the major point is that the compounds should be synthetically feasible to enable the experimental screen. Introduced by Enamine Ltd. concept of the REAL (REadily AccessibLe) compounds has shown its applicability to generate a multi-billion chemical space (the REAL space) of synthesizable compounds [7]. The REAL compounds are accessible *via* in-house validated one-pot parallel chemistry applied to in-house available building blocks and reagents [8, 9]. The variety of the synthetic transformations and involvement of a large pool of the reagents allow building diverse libraries introducing new chemotypes for various targets [10, 11].

Validated parallel chemistry protocols result in an enormous expansion of virtual databases [12] and may create a cost-to-screen issue, similar to the one for HTS. Indeed, molecular docking of one million structures is performed on a modern personal computer, but the virtual screening of one billion structures requires a powerful cluster or a cloud. Many small or medium-sized companies and research groups lack access to the above resources. Also, large libraries utilize substantial storage: one million molecules represented as 1,000 conformers per structure to better probe the space (i.e., 1,000,000,000 conformers in total) require a few terabytes of storage.

Nevertheless, molecular docking of large virtual libraries is impossible [11]. Many approaches exist to utilize fewer resources by sampling a chemical space before the screen [13]. In this work, we study a part of the REAL space built utilizing two transformations [8, 14] applying sampling to the output to get a representative small dockable set of compounds. We further demonstrate the advantages of having virtual libraries of synthesizable compounds in the primary hit identification showing the speed and efficacy of such an approach to support fast turnaround. We apply our approach to identify selective binders of BRD4, a member of the bromo- and extra terminal domain protein family associated with cancer, cardiovascular diseases, inflammation processes, CNS disorders [15–17].

■ Results and discussion

Chemical space

Over the last two years, we proposed two approaches to deliver multimillion chemical libraries based on heterocyclic scaffold-like cores [8, 14]. The chemical space generated based on the approaches meets the REAL paradigm:

1) a wide range of starting reagents is available, amines and carboxylic acids, both classes contribute greatly to the universe of the commercially available building blocks and reagents;

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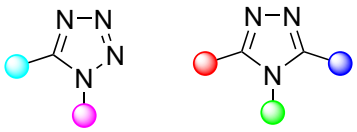

1 **2**

Figure 1. A potential molecule expansion for the REAL tetrazoles (**1**) and the REAL triazoles (**2**)

2) one-pot procedures are compatible with the parallel chemistry setup;

3) the optimization of synthetic procedures affords medium to high synthesis success rates.

A heterocyclic central core acts as a rigid linker. The molecules in the library extend in two or three directions in the case of tetrazole- or triazole-based structures, respectively (Figure 1).

For this study, we generated a virtual library of the REAL compounds from these two reactions and commercially available in-stock reagents. We focused on the drug-like space limited by Veber rules [18, 19], which resulted in 221,000,000 individual REAL molecules (physicochemical profiles and the quality assessment of the library are given in the Supporting Information File). The sampling of the library by grouping resulted in reduction to 100,000 clusters. The centroids of the clusters were further utilized in virtual screens against BRD4.

Target preparation and molecular docking

For target preparation, we reviewed information from UniProt [20], ChEMBL [21], and Protein Data Bank [22] for all known BRD4(1) protein-ligand complexes. After a thorough analysis of the available structures we selected 3U5L(08K) [23] complex to model the 3D structure of the active center (Figure 2).

The binding mode of the ligand in 3U5L(08K) is characterized by a lipophilic sandwich of its fused core between residues Val87, Leu92, Leu94, and Tyr97 from one side, and Phe83 and Ile146 from the other side of the binding pocket (Figure 2). The van der Waals interaction energy in this host-guest pair is dominant. This observation positively correlates with the molecular size; hence, a higher false-positive rate by the scoring function can be expected for larger compounds. To address this issue, we manually inspected the ultimate set of top-scoring molecules received after the virtual screen. The key electrostatic interaction is a hydrogen bond between the benzotriazepine moiety of the ligand with the highly conserved Asn140, a typical feature among bromodomain inhibitors as acetyl-lysine mimics. Water molecules found at the bottom of the pocket are non-displaceable in all known structures. Compound 08K also occupies the hydrophobic sub-pocket located in between Trp81, Pro82, Met149, and Ile146 residues, an important region for the ligand design to gain potency.

Additional justification for a protein-ligand complex was from the analysis of multiple molecular dynamics (MD) simulations of 55 compounds with different anchor fragments starting from their docked poses into BRD4(1). The study allowed us to evaluate the main binding interactions,

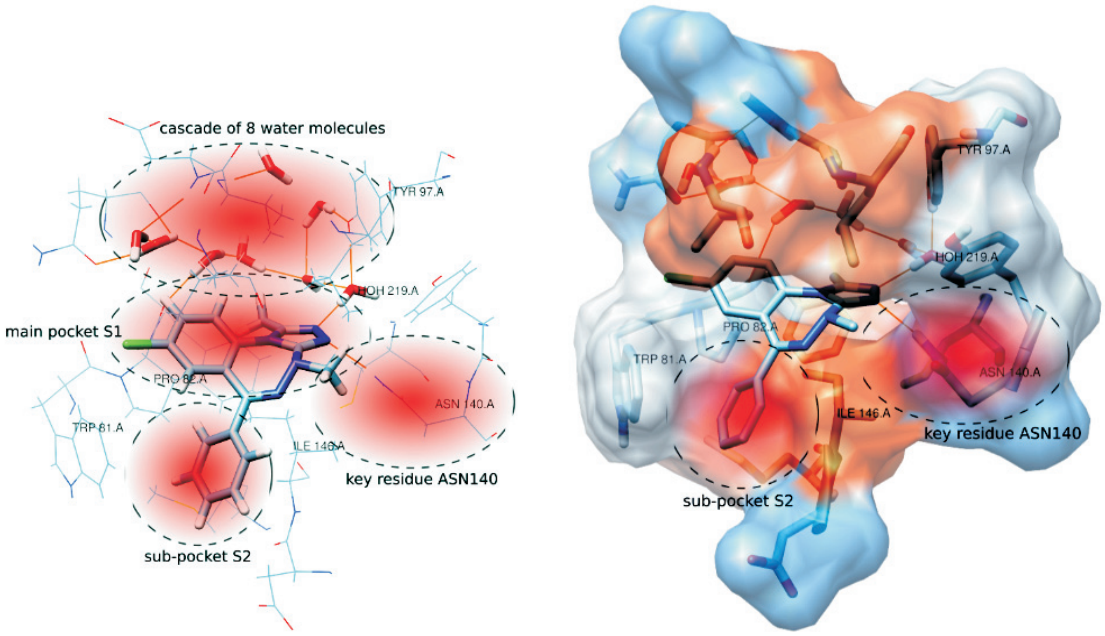

Figure 2. A representation of the active center of BRD4(1) with the bound ligand (PDB: 3U5L, ligand 08K)

for example, the stability of the hydrogen bond with Asn140 [24]. The MD simulations revealed that the binding modes of some of the chemotypes selected were not stable. Ligands are known to induce differences in shape among crystal structures and this, in turn, changes the structure and binding energy predictions of any docking protocol. To increase the chances of successful identification of new diverse chemotypes for BRD4(1), we used the X-ray crystal structure model with a co-crystallized ligand 08K. This protein-ligand complex resembles all characteristic features of BRD4(1) and was experimentally resolved (PDB: 3u5l) [23].

There are a few observations that should be attention paid to:

- *Asn140 acts as a key residue*

As previously discussed [24, 25], there are conserved interactions within the BRD4(1) binding site. Comparison of the crystal structure of BRD4(1) (3U5L) in a complex with a benzotriazepine-like ligand (08K) to any of the known BRD4 protein-ligand complexes reveals the same conserved interactions between the protein and the bound ligand. Figure 2 shows this contact as an orange solid line (2.16Å) representing a hydrogen bonding interaction with the NH_2-group (Asn140), while the residues are shown as sticks.

- *A key water molecule at Tyr97*

One water molecule (HOH219, at 2.059Å from the nitrogen of 08K) is conserved across most of the chemotypes known. It plays an important role by bridging between the ligand and the Tyr97 residue through hydrogen bonds. This water molecule is in the chain of 8 commonly conserved water molecules and is the closest water to Asn140.

- *A cascade of 8 water molecules retaining across the known X-ray structures of BRD4(1)*

There are conserved water interactions across various known X-ray structures. Especially, eight water molecules are retained in most BRD4(1) structures. It appears to be unlikely to alternate such an ensemble of interconnected water molecules with a ligand fragment. Therefore, these water molecules (Figures 3 and 4) were preserved in our simulations. Elimination of all other water molecules allowed to study new interactions.

- *A hydrophobic sub-pocket with a preferable π-π interaction*

As observed previously, an important feature of the potentially active molecules along with the binding to Asn140 would be an occupation of the sub-pocket S2 being close to Trp81, Pro82, Met149, and Ile146 residues (Figure 3).

The molecular docking study and subsequent selection of top-scoring molecules have identified 40 potential BRD4(1) binders. One of the molecules is shown in Figure 3. Compound **1**{32,31,15} is remarkably well accommodating the main pocket S1 with a propyl group terminated with a triazole ring, while the central triazole moiety occupies an important sub-pocket S2. Furthermore, the other part of **1**{32,31,15} gains some extra energy from a T-shape π-π contact with Trp81.

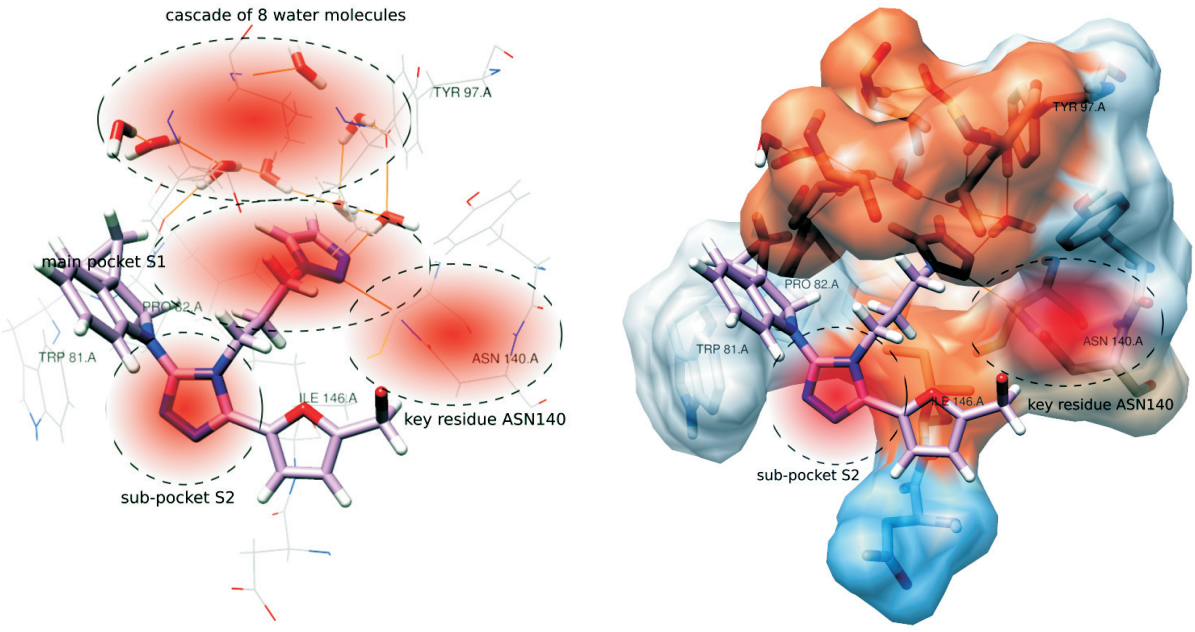

Figure 3. A docking pose of the ligand **1**{32,31,15} in BRD4(1). The molecule of the ligand has been partially truncated for better representation and clarity

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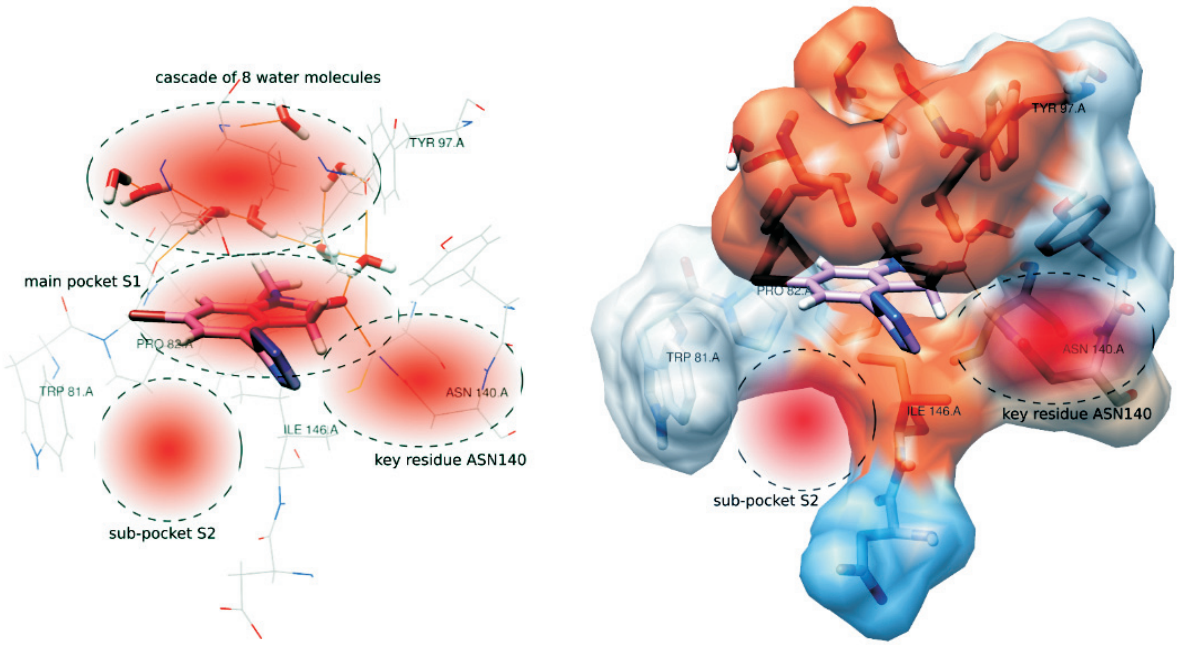


Figure 4. A docking pose of the ligand **1**{13,13,6} in BRD4(1). The molecule has been partially truncated for better representation and clarity

Another molecule from the set, **1**{13,13,6}, interacts with the active site by engaging its carbonyl group into hydrogen bonding interactions with (a) the key residue Asn140 and with (b) a water molecule bridging over to Tyr97. These interactions are sufficient to keep the molecule in the pocket and leave the sub-pocket S2 vacant.

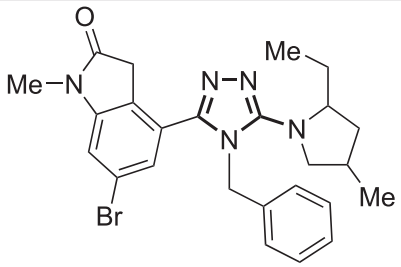
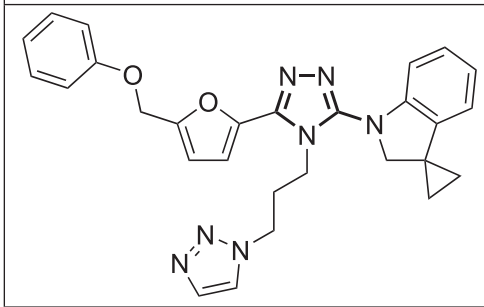
Chemical synthesis and thermal shift assay

All top-scoring molecules were selected for the synthesis. The synthesis performed as described [8, 14] resulted in 29 compounds (72.5% success rate). The list of the compounds obtained with docking scores is given in the Supporting Information File.

All the compounds synthesized were screened against BRD4(1) in the thermal shift assay (the detailed information is given in the Experimental part). Two compounds, **1**{13,13,6} and **1**{32,31,15} (Table), showed a noticeable shift (Figure 5).

A counter screen experiment against the non-related proteins (SIRT1 and Abl SH2) confirmed

Table. Hit molecules after a single round of the screening

Structure	Identifier	Docking score	Distance to the nearest neighbor binder for BRD4	*dTmD RSD* BRD4(1)
	**1**{13,13,6}	-41.58	0.72 [26]	0.76
	**1**{32,31,15}	-40.04	0.82 [27]	0.75

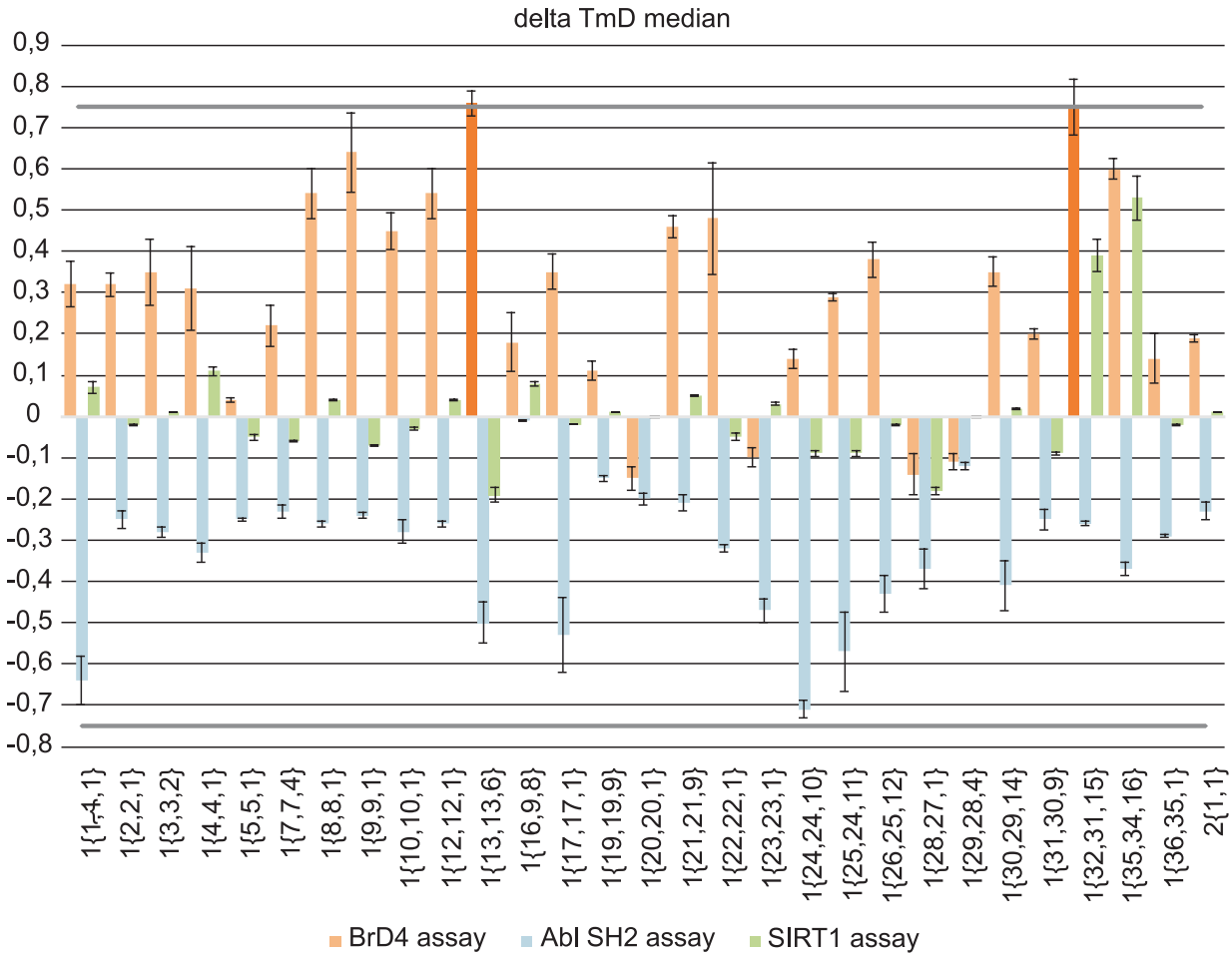


Figure 5. Values of median *dTm* for the screened compounds against BRD4 (**Orange**); Abl SH2 (**Blue**), and SIRT1 (**Green**)

the selectivity of the above hits towards BRD4 showing no significant influence on the stability of the latter proteins. Therefore, the hit rate for the screen was 6%.

Hit expansion from the generated chemical space

One of the advantages of our approach is that the analogs for hit expansion for the hits tested are readily accessible from the very same chemical space the hits were selected from. For **1**{13,13,6}, there are 185 ready-for-synthesis compounds with a Tanimoto similarity over 0.65 (MFP2). For **1**{32,31,15}, we have found over 140 analogs by the same metric. The analogs are available for a wet screening within 3–4 weeks.

Discussion

The drug discovery is an expensive process, and speeding up its stages is very important [28]. The approach proposed will allow finding hit molecules from a large dense chemical space (Figure 6). The creation of the space according to the criteria described gives an access to high-quality molecules (Step 1). Wise sampling supports the diversity of chemotypes and full coverage of the space (Step 2). The sampled subset is screened faster and more economically compared with the full space (Step 3). The synthesis of the molecules selected results in more than 70% of compounds delivered for a wet assay within 3–4 weeks (Step 4). Once found the hit series can be expanded with 1,000–10,000 make-on-demand analogs available for a wet screening in 3–4 weeks (Steps 5–8). The latter steps can be iterated to find more potent analogs compared with the initial hits. If successful, the approach will allow speeding up the hit-to-lead or even lead optimization stages.

■ Conclusions

We evaluated a methodology for obtaining starting points at the initial stages of the drug discovery. The approach proposed is suitable for a rapid (6–8 weeks) hit expansion from a dense chemical space. When iterated the approach can be applied to the hit to lead stage speeding it up by the application of the fast synthesis and hit evaluation steps.

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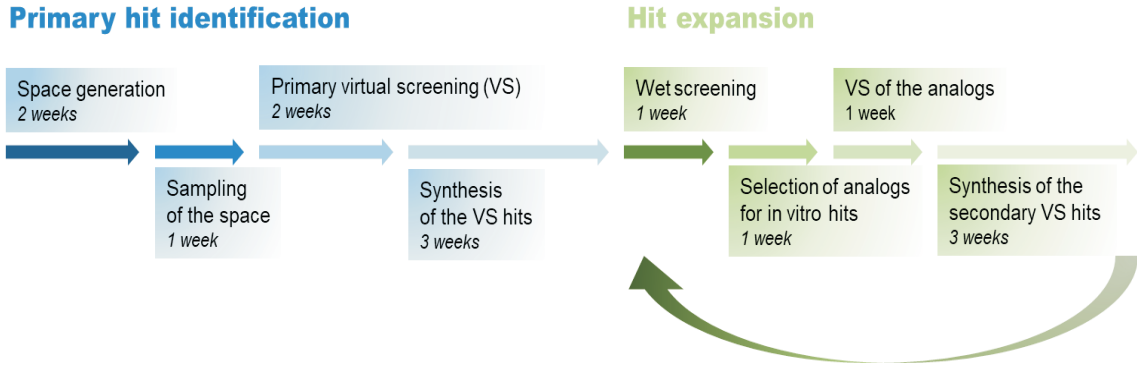

Figure 6. General workflow

■ Experimental part

Chemical space generation, cutoffs, clustering

The chemical space was generated from the in-stock available building blocks using reactions of the formation of aminotetrazoles and aminotriazoles [8, 14]. The whole chemical space comprised 674 million unique compounds.

Narrowing down the space by utilizing "Drug-Likeness" and "Veber Rules" criteria [19, 30], as well as using substructure filters (e.g., PAINS as implemented in RDKit) [31] resulted in 221 million compounds. This subset of molecules was further diversified utilizing Morgan Fingerprints (MFP2) in conjunction with Tanimoto Similarity metrics. For this purpose, the following functions implemented in the RDKit package for python were used: LazyBitVectorPick() from rdkit.SimDivFilters.MaxMinPicker().

Ultimately, a diverse subset of a hundred thousand dissimilar molecules was subjected to the molecular docking study.

Docking

In our virtual screens, we have used Dock [32]. Reference active ligands, decoys, and the diversified set of triazole/tetrazole derivatives were docked into the BRD4 binding site of the **3U5L** protein structure using DOCK 3.7.3 [33].

The docking program places pre-generated flexible ligands into the binding site (specified by matching spheres) *via* atomic superposition of each prob molecule on matching spheres (generated based on the crystallographic ligand), thus, representing favorable atomic positions of each conformer prior to scoring. The general pipeline for placing matching spheres into the binding site is to use the crystallographic ligand heavy atoms as the first set of spheres and then to add nearby spheres generated by SPHGEN routine until a set number of matching spheres is reached. By default, forty-five matching spheres were used in the template receptor structure. The resulting ligand poses were scored by adding up the receptor-ligand electrostatic interaction energies to van der Waals interaction energies, and then corrected for context-dependent ligand desolvation.

The receptor structure was protonated using a Reduce program [34]. Partial charges from the united-atom AMBER [35] force field were used for all receptor atoms. The grid to evaluate protein-ligand electrostatics was pre-calculated using a QNIFFT [36, 37] program. CHEMGRID[38] was used to make the van der Waals grid using an AMBER forcefield for the receptor. SOLVMAP [39] was used to calculate a grid for the evaluation of the ligand desolvation term.

Synthesis

Experimental procedures applied for the synthesis of compounds were described [8, 14]. The synthesis was completed in 3 weeks, with 29 of 40 selected compounds synthesized and 72.5% success rate.

Assays

All thermal shift assay (TSA) experiments with BRD4 protein were performed using a ViiA™7 real-time PCR System equipped with a 384-well heat block (Applied Biosystems, Waltham, MA, USA). The optimal buffer composition for the TSA procedure was determined as described previously [40]. The buffer consisting of 50 mM Phosphate-Na, 100 mM NaCl, pH 7.5 was selected for the BRD4 screening in the previous study [41]. A purified BRD4 protein was pre-mixed with SYPRO Orange dye (Thermo Fischer Scientific, Cat. S6650, 5000x stock, Waltham, MA, USA) to prepare a master mix in the concentrations of 4 μM protein and 6× dye. The compounds tested were added to the protein- at 40 μM in the final DMSO concentration of 2% and pre-incubated at room temperature

for 15 min in MicroAmp® optical 384-well reaction plates (ThermoFisher, Cat. 4309849, Waltham, MA, USA) sealed with an optical sealing film (ThermalSeal RT2, Excel Scientific, Cat. TS-RT2, Victorville, CA, USA). After the compounds preincubation with the protein the dye was added to all wells making the final concentration of 6×. The volumes of all reaction mixtures were 10 μL (4 μg BRD4 per well). The thermal scanning was performed by raising the temperature to 40°C at 1.6°C/min without the signal detection followed by 40°C to 90°C temperature ramp at 0.05°C/s with the constant fluorescence intensity reading at 2-sec intervals using an EX470/EM623 nm filter set.

Assay procedures for SIRT1 and Abl SH2 were the same as for BRD4, with exception of the assay buffer composition, protein, and dye concentration, which were used. Additionally, the thermal ramp was adopted individually for each protein.

While testing the compounds with SIRT1 the reaction was kept in the following assay buffer: 20 mM Tris pH 7.5 and 50 mM NaCl. The protein and dye were added to the final concentration of 1.3 μM and 8×, respectively. The thermal scanning was performed by raising the temperature to 35°C at 1.6°C/min without the signal detection followed by 35°C to 75°C temperature ramp at 0.05°C/s with the constant fluorescence intensity reading at 2-sec intervals using an EX470/EM623 nm filter set.

During the assessment of compounds with Abl SH2 the reaction was kept in the following assay buffer: 50 mM Tris, 150 mM NaCl, and 2 mM $MgCl_2$. The protein and dye concentrations were 1 μM and 8×, respectively. The thermal scanning was performed by raising the temperature to 40°C at 1.6°C/min without the signal detection followed by 40°C to 75°C temperature ramp at 0.05°C/s with the constant fluorescence intensity reading at 2-sec intervals using an EX470/EM623 nm filter set.

The peak of the first derivative for the fluorescence curve was used to determine the melting temperature (T_m). The averaged T_m values for the control wells (64 wells per plate) containing only the protein, dye, and DMSO (1%) were used as a reference point to determine the melting temperature shifts (ΔT_m).

We selected the hits based on the following selection criteria [40]:

$$|dTmD| > 3*TmD\ SD\ of\ DMSO\ control$$

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