Sulfur(VI) Fluoride Exchange Chemistry in Solid-Phase Synthesis of Compound Arrays: Discovery of Histone Deacetylase Inhibitors

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Supporting Information Placeholder

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ABSTRACT: Multistep synthesis performed on solid support is a powerful means to generate small molecule libraries for the discovery of chemical probes to dissect biological mechanisms as well as for drug discovery. Therefore, expansion of the collection of robust chemical transformations amenable to solid-phase synthesis is desirable for achieving chemically diverse libraries for biological testing. Here we show that sulfur(VI) fluoride exchange (SuFEx) chemistry, exemplified by pairing phenols with aryl fluorosulfates, can be used for solid-phase synthesis of biologically active compounds. As a case study, we designed and synthesized a library of 84 hydroxamic acid containing small molecules, providing a rich source of inhibitors with diverse selectivity profiles across the human histone deacetylase enzyme family, which is a validated drug target. Among other discoveries, we identified a scaffold that furnished inhibitors of HDAC11 with exquisite selectivity in vitro and a selective inhibitor of HDAC6 that was shown to bind this target enzyme selectively over HDAC8 in cells, using cellular thermal shift assays (CETSA). Our results encourage the further use of SuFEx chemistry for the synthesis of diverse small molecule libraries, provides insight for future design of selective HDAC inhibitors, and show that CETSA can be applied for evaluation of cellular target engagement of HDAC inhibitors.

INTRODUCTION

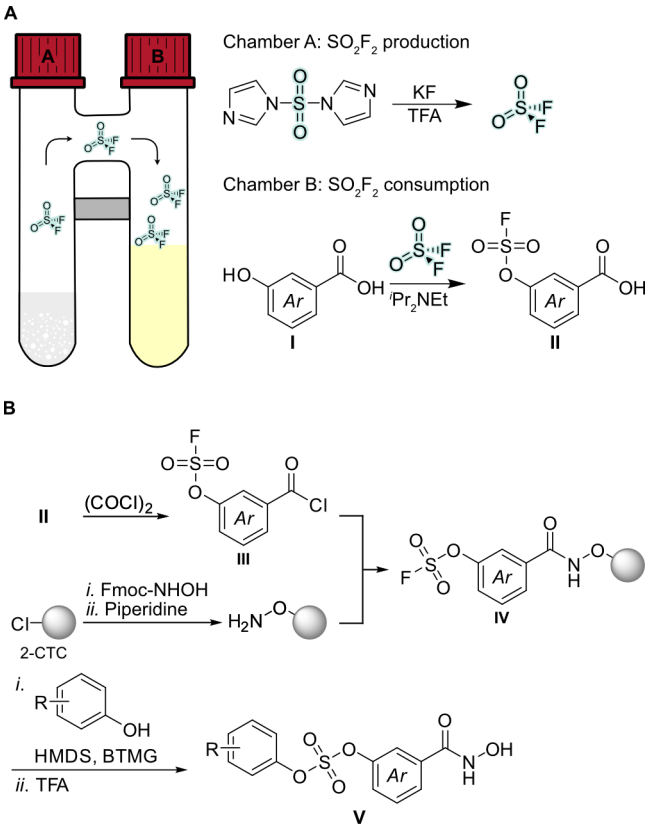
Drug discovery campaigns often rely on the generation and screening of collections of chemical compounds during both hit identification and lead optimization.[1] The development of solid-phase synthesis technology, originally for peptide synthesis,[2] enabled the chemical synthesis of libraries of peptides[3, 4] and led to the development of combinatorial chemistry.[5-8] The synthesis of compound libraries rapidly evolved to include non-oligomeric chemotypes[9-12] and attention has since been dedicated to the importance of the structural diversity of the compound collections for success in identifying biologically relevant ligands.[13-15] Still, most small molecule compound libraries are generated by using a limited set of chemical transformations: amide coupling, aromatic nucleophilic substitution, reductive amination, and transition metal catalyzed cross-coupling reactions.[16] These choices may, at least in part, reflect the requirements that reactions used for library synthesis need to be reliable, high yielding, and have broad functional group tolerance. As such, sulfur(VI) fluoride exchange (SuFEx) chemistry, which has found considerable use in covalent chemical probe design,[17-23] has been widely developed[24, 25] and included as a viable addition to this toolbox,[26-28] since it was coined in 2014.[29] In this work, we showcase the extension of SuFEx chemistry – for generation of biologically active small molecules – to solid-phase synthesis, which enables the rapid generation and handling of large compound libraries.

RESULTS AND DISCUSSION

As a case study, we chose to investigate the discovery histone deacetylase (HDAC) inhibitors with novel selectivity profiles, by combining a selection of resin bound SuFEx hubs with a range of phenols in a parallel synthesis format. Histone deacetylases are therapeutically relevant drug targets,[30, 31] and inhibitors that bind to the active sites of these enzymes generally contain a zinc-binding group, a linker that mimics the side chain of a lysine residue, and a capping group that interacts with the surface of the enzyme.[32] We therefore designed a strategy based on a number of aryl fluorosulfate building blocks, prepared in a two-chamber reactor (**II**; Scheme 1A).[33-35] These building blocks could then be converted to the corresponding acid chlorides (**III**, Scheme 1B) and coupled to a solid

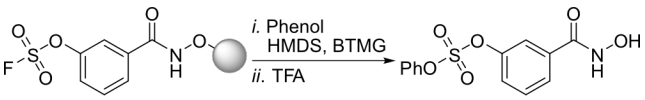
supported hydroxyl amine to give resin bound hydroxamic acids, which have previously been applied for discovery of HDAC inhibitors.[36-41] The resin-bound hydroxamates prepared in this study (**IV**) displayed the aryl fluorosulfate SuFEx hubs for functionalization with a panel of phenols, to give diverse hydroxamic acid containing compounds (**V**) upon cleavage from the resin (Scheme 1B).

Scheme 1. A) Synthesis of aryl-fluorosulfates by utilizing a two-chamber system for ex situ formation of SO_2F_2. B) Strategy for on-resin, accelerated SuFEx chemistry to generate compound arrays.



The aryl fluorosulfate SuFEx functionality was strategically chosen due to its latent reactivity profile to enable the manipulations required for preparing and handling the resins. Importantly, we then envisioned that the recently developed conditions for accelerated SuFEx chemistry,[42] could be adapted to catalyze the functionalization of our resins (**IV**) [using 2-*tert*-butyl-1,1,3,3-tetramethylguanidine (BTMG; "Barton's base") and hexamethyldisilazane (HMDS)].

Table 1. Screening of reaction conditions for on-resin SuFEx reaction. Monitored by HPLC after cleavage from resin.



entry	solvent	phenol	time	temp.	conv.[a]
1	MeCN	PhOH	2 h	20 °C	4 %
2	MeCN[b]	PhOH	2 h	65 °C	52 %
3	CH_2Cl_2	PhOH	0.5 h	20 °C	12 %
4	CH_2Cl_2	PhOH	2 h	20 °C	9 %
5	DMF	PhOH	0.5 h	20 °C	9 %
6	DMF	PhOH	2 h	20 °C	47 %
7	DMF	PhOH	0.5 h	65 °C	19 %
8	DMF	PhOH	2 h	65 °C	75 %
9	DMF	PhOH	16 h	65 °C	>99 %
10	DMF[c]	*p*-CF_3-PhOH	16 h	65 °C	>99 %
11	DMF[c]	*o*-*i*Pr-PhOH	16 h	65 °C	>99 %

Conditions: phenol (5.0 equiv), HMDS (5.0 equiv), and BTMG (1.0 equiv) relative to the resin loading (2.6 μmol scale), solvent (200 μL). [a]Conversion given as $AUC_{product} \times (AUC_{product} + AUC_{unreacted\ ArOSO2F})^{-1}$ measured by HPLC at 215 nm. [b]Solvent evaporated during the reaction time. [c]20 μmol scale.

Our initial attempts to use acetonitrile as the solvent, as originally reported by the Moses and coworkers,[42] led to poor swelling of our polystyrene based resins. Further, the heating required for product formation caused rapid evaporation of the solvent, prohibiting extended reaction times (Table 1, entries 1 and 2). Polystyrene-based resins have excellent swelling properties in dichloromethane, but low conversion was also observed in this solvent at room temperature (entries 3 and 4). We therefore investigated *N,N*-dimethylformamide (DMF) and found that elevated temperature (65 °C) for two hours gave acceptable conversion (entries 5–8), while extended reaction time (16 h) at this temperature gave full conversion (entry 9). These optimized conditions also gave full conversion when using a sterically hindered or an electron deficient phenol (entries 10 and 11). For the cleavage step, we found that dilute trifluoroacetic acid (TFA) was superior to the milder 1,1,1,3,3,3-hexafluoro-isopropyl alcohol, and the resulting crude chromatograms revealed minor impurities from the catalysts.

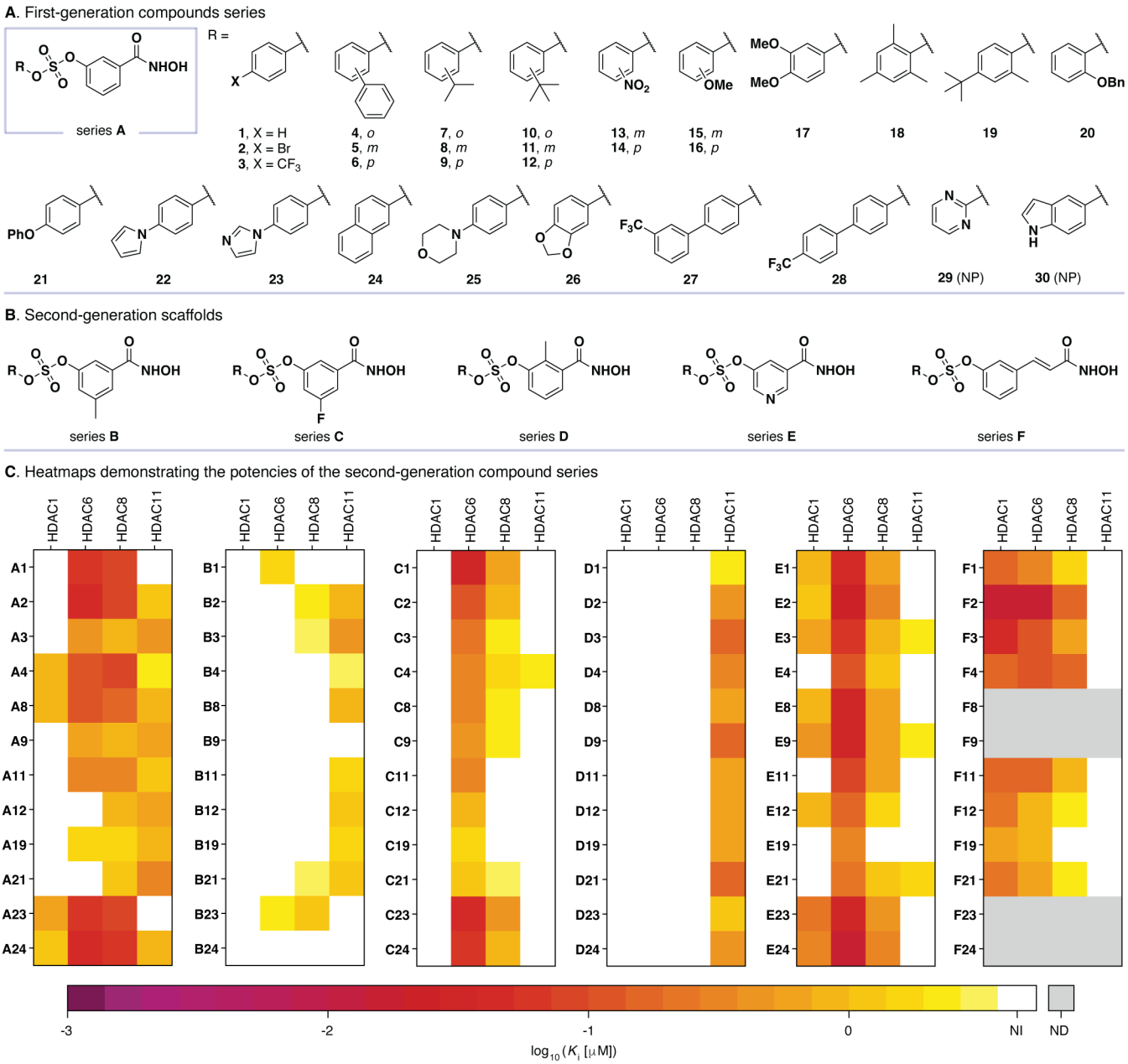


Figure 1. (A) Structures of compounds **A1–A30** (for compound synthesis, see Supplementary Figs. S1 and S2); NP, no product formed. (B) Structures of scaffolds **B–F** for the second-generation compound series. (C) Heat maps representing K_i values of selected first-generation members and the full second-generation series against HDAC1, HDAC6, HDAC8, and HDAC11. The K_i values are calculated, using the Cheng-Prusoff equation, from IC_{50} values determined by at least two individual end point assay, performed in duplicate. See Supplementary Figures S4–S9 for full dose-response curves and Supplementary Tables S1 for IC_{50} and calculated K_i values. NI, no inhibition as determined by <50% inhibition at 5 μM inhibitor; ND, not determined as compound was left out of the series.

The on-resin functionalization thus appears to reduce the degree of Lossen rearrangement, which is a known side reaction between hydroxamic acids and aryl fluorosulfates.[43] Final compounds were still purified by preparative high-performance liquid chromatography (HPLC) using a short gradient to give purities >95%.

For the first-generation compound series, we chose scaffold **A** in combination with a selection of 30 phenols/hydroxy-aryl compounds (R-OH with R = **1–30**; Figure 1A). Final products **A1–A28** were all successfully prepared and only the reactions with pyrimidin-2-ol (**29**) and 5-hydroxyindole (**30**) were unproductive according to LC-MS analysis of the cleaved crude material; presumably due to the poor nucleophilicity of these building blocks. The potencies of the initial compound series were then evaluated against a selection of the human zinc-dependent

HDACs (HDAC1–11). We selected HDACs 1, 6, 8, and 11 as targets for inhibition to include representatives of sub classes I, IIb, and IV. The HDAC6 was considered particularly interesting target due to its recent status as an orphan drug target by the US Food and Drug Administration. Also, HDAC8 plays a potential role in several cancers, including T-cell lymphoma,[44] and HDAC11 is of interest due to the under representation of tool compounds available to study its biological function.[45]

All compounds **A1**–**A28** exhibited limited potencies against HDACs 1 and 11 but generally inhibited HDACs 6 and 8 (see Supplementary Figure S3 for heat map and Supplementary Table S1 for K_i values). Potent inhibition of both HDACs 6 and 8 were observed, for example for compounds **A22** (K_i = 17 nM against HDAC6) and **A17** (K_i = 22 nM against HDAC8), respectively. However, modest selectivity below 10-fold were observed between the inhibition of the two enzymes.

In the next iteration of synthesis and biochemical evaluation, we focused on altering the structure of the scaffold to give series **B**–**F** (Figure 1B). We selected phenols to represent ones from potent HDAC6 and 8 inhibitors (**A1**, **A2**, and **A23**), from compounds that inhibited HDAC11 (**A3**, **A11**, **A12**, **A19** and **A21**), and finally from non-selective inhibitors (**A4**, **A8**, **A9**, and **A24**) (Figure 1C). Combining these phenols with the five scaffolds provided the series **B**–**F**, incorporating substituents in the 2- and 5-position of the scaffold (**B**–**D**) and including a *meta*-substituted pyridine ring (**E**). The series **F** was based on 3-hydroxy-cinnamic acid, resembling the clinically approved, non-selective HDAC inhibitor belinostat (Beleodaq®).[31] When testing these series against the same selection of enzymes as selected in the initial screen, the series **B** compounds showed a general decrease in potency against HDACs 6 and 8, while some potency was retained against HDAC11 (Figure 1C). Substituting the methyl group in **B** for a fluorine atom in **C**, resulted in retained potency against HDAC6 compared to the series **A**, but with improved selectivity due to lower potencies observed against HDAC8. For example, the compound **C1** exhibited 20-fold selectivity for HDAC6 over HDAC8, while the compound **A1** was equipotent against these two enzymes (Supplementary Table S1). In series **D**, where a methyl group was introduced in the 2-position of the scaffold, a striking loss of activity was observed against both HDAC6 and 8. On the other hand, a general increase in potency against HDAC11 was recorded and compounds **D3**, **D9**, and **D21** inhibited this enzyme with K_i values in the 150–190 nM range (Supplementary Table S1). Thus, these compounds exhibited excellent isoform selectivity with no inhibition of the other tested HDACs at concentrations up to 5 μM. Interestingly, a recent study from Wang and coworkers also reported HDAC11-selective inhibitor with an *ortho*-substituted benzohydroxamic acid,[46] suggesting that this motif may offer a general avenue for the future development of optimized inhibitors of HDAC11.

The pyridine containing series (**E**), like series **C**, generally furnished inhibitors with selectivity for HDAC6, but with decreased selectivity over to other isoforms (Figure 1C). Compound **E24** was identified as the most potent compound of the library with a K_i value of 14 nM against HDAC6 and ~20-fold selectivity over HDAC8 (Supplementary Table S1). The series **F** was devoid of HDAC11 inhibitors and instead provided several compounds with potency against all the remaining three HDACs (1, 6, and 8), consistent with the activities of the known cinnamic hydroxamate, belinostat.[31]

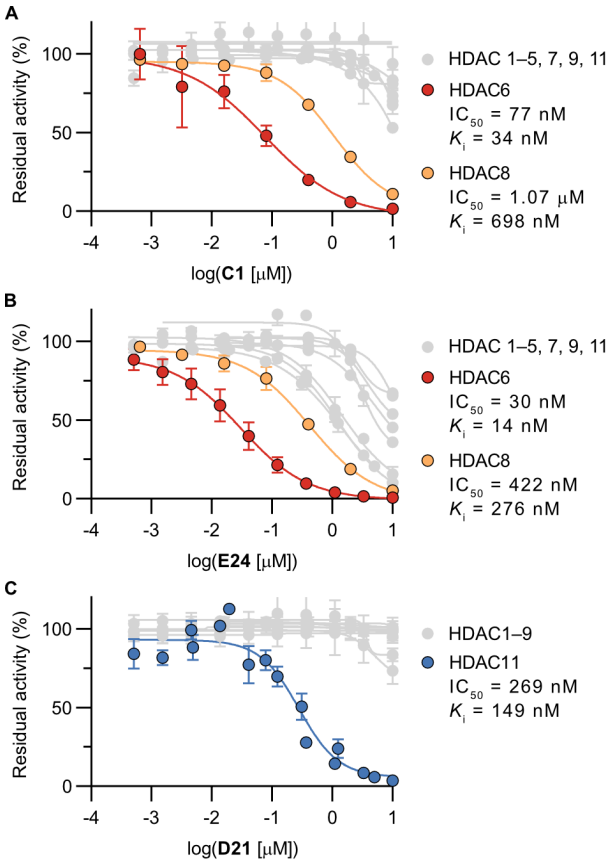


Figure 2. Selectivity of selected compounds against HDACs 1–9 and 11. (A) IC_{50} curves for **C1**. (B) IC_{50} curves for compound **E24**. (C) IC_{50} curves for compound **D21**. The K_i values are calculated from IC_{50} values determined by at least two individual end point assays performed in duplicate, by using the Cheng-Prusoff equation. See Supplementary Figures S10, S11 and Supplementary Table S2 for additional data.

To investigate the selectivity of selected inhibitors across a broader selection of HDAC isoforms, we picked eight compounds with different selectivity profiles in the initial screen (**A10**, **C1**, **D21**, **E3**, **E23**,

E24, **F2**, and **F3**) (Supplementary Figure S11 and Supplementary Table S2). This analysis revealed inhibitors with selectivity for HDAC6 (**C1**, **E3**, **E23**, **E24**) (Figure 2A,B), HDAC8 (**A10**), and HDAC11 (**D21**) (Figure 2C). Further, scaffold **F** furnished non-selective ones (**F2** and **F3**) that target all enzymes except HDAC11 and class IIa isoforms, which have been reported to have non-catalytic function and are thus poorly targeted by hydroxamic acids.[47, 48]

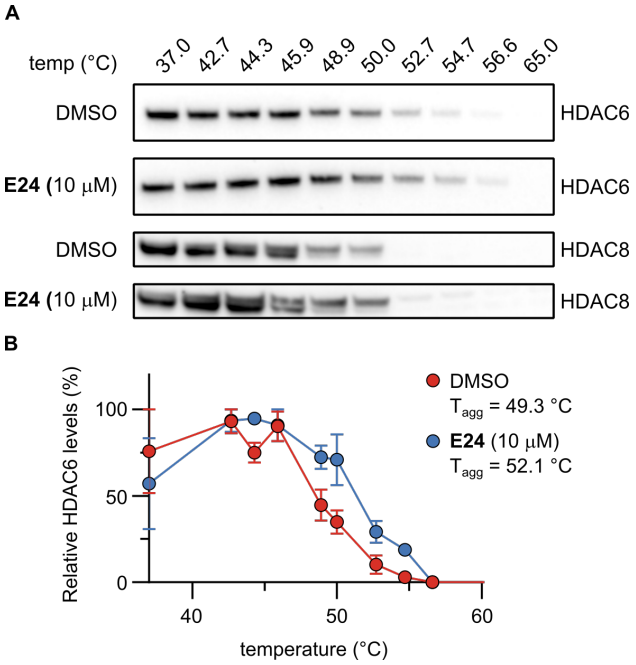


Figure 3. Cellular target engagement of compound **E24**. (A) Representative immunoblots for the thermal shift of HDAC6 and HDAC8 in HEK293T cells after 2 h treatment with inhibitor **E24** (10 μM) at temperatures ranging from 37–65 °C, comparted to DMSO control. (B) Plots of the data and calculated T_{agg} values (n = 2; see the Supplementary Figure S12 for full blots and replicates).

With our novel series of inhibitors, exhibiting a wide range of selectivity profiles against recombinant enzymes in biochemical assays in vitro, we were interested in demonstrating target engagement and determining selectivity in cells. For this purpose, we chose to use evaluation by Western blot based cellular thermal shift assays (CETSA),[49, 50] which we have previously used for investigating inhibitors targeting sirtuins (NAD^+-dependent class III HDACs).[51-54] Because HDAC11 has very low expression levels in most cell lines, we were not able to produce viable melting curves for this enzyme. Instead, we chose the most potent HDAC6 inhibitor of our collection (**E24**), which represented the other selectively targeted enzyme isoform according to the biochemical assays. With HDAC8 being the closest targeted isoform (Figure 2B), we also evaluated the thermal shift of this enzyme. In cultured human embryonic kidney (HEK)293T cells we observed a thermal shift of HDAC6 of ~3 degrees upon treatment with compound **E24**, relative to DMSO control and no shift was observed for HDAC8 (Figure 3). These data strongly suggest that the inhibitor **E24** selectively binds to and stabilizes HDAC6 over HDAC8, rendering it a selective HDAC6 inhibitor in live cells.

CONCLUSION

In summary, we have developed a strategy for using SuFEx click chemistry on solid support to generate arrays of biologically active compounds. In this case study, we successfully applied the aryl fluorosulfate functionality as a hub for diversification during solid-phase synthesis of potential HDAC inhibitors. By parallel synthesis, we prepared a library of 84 diverse compounds, which furnished potent HDAC inhibitors with a broad range of selectivity profiles. Thus, the present work demonstrates SuFEx chemistry as an addition to the existing arsenal of reactions for diversification of small molecule libraries on solid phase. These results argue for the future application of additional SuFEx reactions on solid support and we further envision that our data should serve as encouragement for similar expansion of the chemical transformations applied for the synthesis of DNA encoded libraries.

The biochemical evaluation of the library against recombinant HDAC enzymes furnished a structure–activity relationship (SAR) study, which identified key features of importance for the targeting of individual HDAC isoforms. Particularly, we successfully identified novel isoform selective inhibitors against HDACs 6 and 11, of which the former mentioned was confirmed by CETSA to engage the desired target with selectivity in HEK293T cells. Finally, cellular thermal shift melting curves were thus confidently demonstrated as a method for evaluation of cellular target engagement of HDAC inhibitors.

METHODS

General procedure 1. Aryl fluorosulfate formation.[35] Chamber A of a two-chamber reactor (COware gas reactor; Sigma #STW1 (1 mmol) or #STW5 (5 mmol) was charged with 1,1'-sulfonyldiimidazole (1.5 equiv) and potassium fluoride (4.0 equiv). Chamber B was charged with the desired phenol (1 equiv) followed by H_2O–MeCN (1:1, 4 mL) and added iPr$_2$NEt (3.0 equiv). The two chambers were sealed and TFA (0.5 mL per mmol phenol) was added by injection through the septum of chamber A. The reaction was stirred for 16 h at room temperature before the caps were removed and the reaction was stirred for another 15 minutes to ensure that all sulfuryl fluoride was vented out of the fume hood. Next, the content of chamber B was transferred to a

round-bottomed flask and volatiles were removed under reduced pressure. The residue was acidified to pH 2 with aqueous HCl (1 M) and extracted twice with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give the crude residue.

General procedure 2. Formation of acid chloride and resin functionalization. Aryl fluorosulfate modified carboxylic acid (1 equiv) was dissolved in anhydrous CH_2Cl_2 (10 mL/mmol). The solution was stirred on an ice bath and oxalyl chloride (2 equiv) was added followed by DMF (two drops). The reaction was allowed to warm up to room temperature and stirred for 2 hours before volatiles were removed under reduced pressure to give the crude acid chloride, which was used immediately in the next step (general procedure 3).

General procedure 3. Loading of 2-chlorotrityl chloride resin and formation of aryl fluorosulfate-functionalized resin. 2-chlorotrityl chloride polystyrene resin was allowed to swell in anhydrous CH_2Cl_2 in a fritted syringe for 15 min. After the solvent was removed, *N*-Fmoc-hydroxylamine (1.5 equiv related to the theoretical loading from the vendor) and *i*Pr_2NEt (5 equiv) in anhydrous CH_2Cl_2–DMF (10:1, 11 mL/g resin) were added and agitated for 2 h at room temperature. The resin was then drained and capped by incubation with CH_2Cl_2–MeOH–*i*Pr_2NEt (17:1:2, 10 mL/g resin, 2 × 15 min); then, washed with CH_2Cl_2 (3 × 1 min), DMF (2 × 1 min), and CH_2Cl_2 (2 × 1 min). The loading was determined spectrophotometrically, quantifying the amount of released fluorene upon cleavage of the Fmoc group from a small sample of dried resin.[55] Loading was typically determined to be 0.5–0.6 mmol/g for different batches of resin. Fmoc deprotection was achieved with DMF–piperidine (4:1, v/v, 9 mL; 2 min; then repeated for 15 min), followed by washing with DMF (5 × 1 min) and CH_2Cl_2 (3 × 1 min). The hydroxylamine functionalized resin was then incubated with the corresponding aryl fluorosulfate-modified acid chloride (1.5 equiv) and *i*Pr_2NEt (6.0 equiv) in anhydrous CH_2Cl_2 (0.2 M of acid chloride) for 90 min at room temperature, followed by washing with DMF (3 × 1 min), MeOH (3 × 1 min), and CH_2Cl_2 (3 × 1 min), before the resin was drained and dried under reduced pressure.

General procedure 4. On-resin sulfur (IV) fluoride exchange (SuFEx) reaction. The aryl fluorosulfate functionalized resin was placed in a fritted syringe and swelled in anhydrous DMF (0.25 mL/10 μmol resin). After 5–10 min, the resin was drained by suction and added indicated phenol (5 equiv), BTMG (1 equiv), HMDS (5 equiv) in anhydrous DMF (0.025 mL/μmol resin) were added and the mixture was agitated for 16 h at 65 °C. The resin was then washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). The hydroxamic acid containing compound was released from the solid support by CH_2Cl_2–TFA (90:10, 0.5 mL, 2 × 15 min). Volatiles were removed under a stream of nitrogen and the crude residue was purified by preparative HPLC.

Optimization of On-Resin SuFEx reaction. Resin A (2.6 μmol) was swelled in anhydrous solvent (200 μL) and reacted with phenol (5 equiv), BTMG (1 equiv), and HMDS (5 equiv) in the desired solvent (200 μL) for the indicated time and temperature. After the reaction, the resin drained and was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) before the product was cleaved with CH_2Cl_2–TFA (90:10, 200 μL, 2 × 15 min). Volatiles were removed under a stream of nitrogen and the crude residue was dissolved in acetonitrile analyzed by ultra-high performance liquid chromatography. Conversion was estimated as the ratio between the area under the curve (AUC) of the product and the released unreacted aryl fluorosulfate in the UV chromatogram at 215 nm (equation 1).

$$\text{conv.} = \text{AUC}_{\text{product}} \times (\text{AUC}_{\text{product}} + \text{AUC}_{\text{cleaved IV}})^{-1} \qquad \text{eq. 1}$$

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge.

Supplementary schemes depicting the synthesis of building blocks and final compounds; figures depicting biochemical assay data, tables of the biochemical data behind the heat maps, experimental methods; chemical synthesis and compound characterization data; as well as copies of HPLC traces, ^{1}H, ^{13}C, and ^{19}F NMR spectra (PDF)

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

2-CTC, 2-chlorotrityl chloride; AUC, area under the curve; BTMG, 2-tert-butyl-1,1,3,3-tetramethylguanidine; CETSA, cellular thermal shift assay; DMF, *N,N*-dimethylformamide; HMDS, hexamethyldisilazane; HDAC, histone deacetylase; HPLC, high-performance liquid chromatography; human embryonic kidney, HEK; LC-MS, liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; SuFEx, sulfur(VI) fluoride exchange; TFA, trifluoroacetic acid.

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