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Machine-assisted synthesis of modulators of the histone reader BRD9 using flow methods of chemistry and frontal affinity chromatography†

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A combination of conventional organic synthesis, remotely monitored flow synthesis and bioassay platforms, were used for the evaluation of novel inhibitors targeting bromodomains outside the well-studied bromodomain and extra terminal (BET) family, here exemplified by activity measurements on the bromodomain of BRD9 protein, a component of some tissue-specific SWi/SNF chromatin remodelling complexes. The Frontal Affinity Chromatography combined with Mass Spectrometry (FAC-MS) method proved to be reliable and results correlated well with an independent thermal shift assay.

The process of drug discovery involves an iterative cycle of design-synthesis-evaluation steps. The information obtained from each cycle is then fed back to the design step, and the loop is repeated until a suitable candidate emerges. Although this approach has largely proven to be successful, drug discovery still relies heavily on exhaustive screening protocols involving the synthesis of a large number of molecules in order to map out the most diverse chemical space relevant to the biological target. Many obstacles must be overcome to achieve an ideal guided discovery process, in which every molecule that is produced takes the researcher one step closer to the desired ligand. For example, the synthesis of a complex molecule requiring multiple steps is still time consuming, labour intensive and expensive. However, with the assistance of automation and other enabling technologies, chemists can provide target molecules rapidly. Nevertheless there is still often a disconnection with the biological assessment of these molecules creating a bottleneck in the process. There is dearth therefore in the methods that can sensibly guide chemists to the next compound in the discovery cycle.

One approach to achieve this "closed-loop" process is to develop an integrated system which encompasses each step of the design, synthesis and functional evaluation elements of drug discovery. For example, we[1] and others[2] have been investigating an integrated platform capable of performing chemical synthesis and subsequent linked biological evaluation. For this, we have reported the use of Frontal Affinity Chromatography (FAC)[3] as a fast analysis protocol suitable for incorporation into our flow chemistry synthesis platform. In this new work, we report the incorporation of a Compact Mass Spectrometer (CMS) to facilitate the analysis, and then use a flow assisted synthesis coupled to the FAC-MS assay for the discovery of BRD9 protein's bromodomain modulators.

The bromodomain is a module found in many proteins[4] including transcriptional co-activators and proteins associated with the remodelling of chromatin.[5] This module primarily recognises specific post-translational protein modifications[6] most notably the ε-*N*-acetylation of lysine residues at the N-terminus of histones.[7]

There are now over 40 proteins containing one or several bromodomains encoded in the human genome which have been grouped into eight families based on their structural similarity.[8] While many bromodomain containing proteins have been linked to disease development, the main effort in chemical biology has been dedicated to the bromodomain and extra-terminal (BET) family, which has been identified as a therapeutic target involved in inflammation,[9] cancer[10] and pathological cardiac hypertrophy.[11]

In 2005, fragments were designed to target the p300/CBP associated factor bromodomain (PCAF BRD).[12] More recently, the potent and selective inhibitors (+)-JQ1 (ref. 13) and I-BET[9] were identified; these compounds share a triazolodiazepine scaffold and inhibit bromodomains from the BET family

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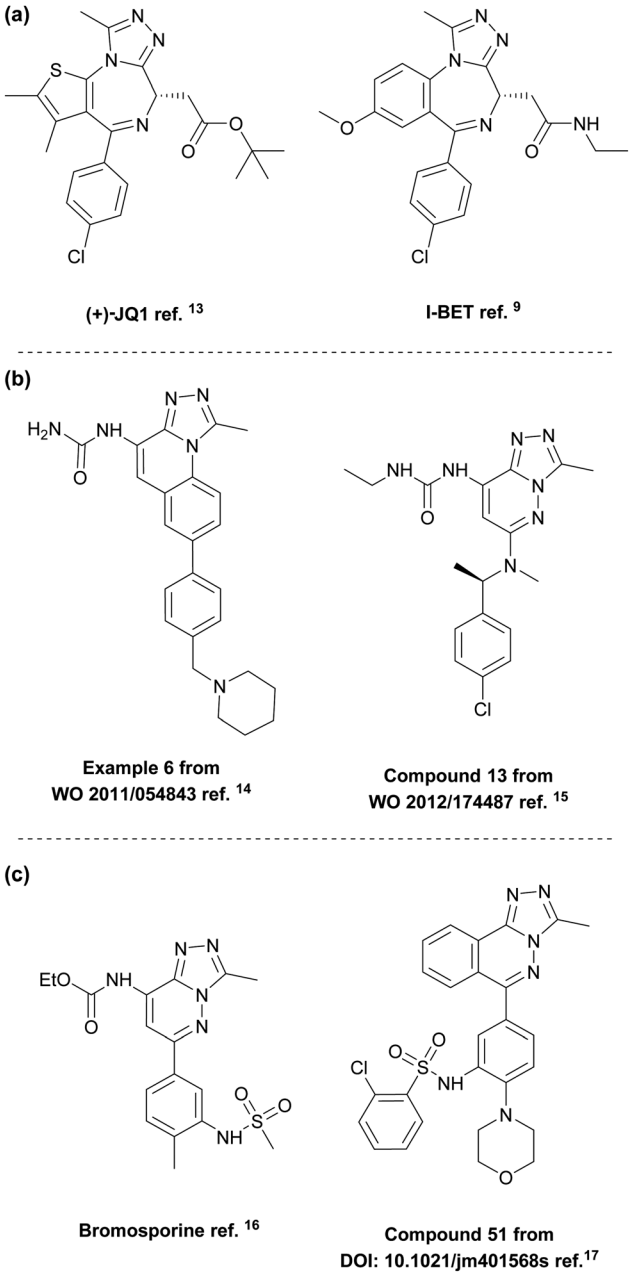


Fig. 1 Structure of triazole containing bromodomains inhibitors: (a) triazolodiazepines; (b) urea containing triazoles; (c) fused triazolopyrazines.

(Fig. 1a). Furthermore, the related urea containing triazoles depicted in Fig. 1b have been patented as BET inhibitors.[14,15] Finally, the recently disclosed fused triazoles presented in Fig. 1c have been reported to inhibit the BET bromodomains as well as other family members such as BRD9, CECR2 and CREBBP.[16,17] However, the biological role of some bromodomains remains poorly understood and a large proportion of them lack any selective inhibitors that could help unveil their individual functions. Amongst these, the bromodomain-containing protein 9 (BRD9) has been shown to be inhibited by several BET inhibitors even though it does not belong to the BET family,[16,17] highlighting the low specificity of some of these inhibitors. However, BRD9's function remains elusive even though its interaction with $H_4K_{12}Ac$, an acetylated histone involved in gene regulatory regions, has been demonstrated.[18] Furthermore, recent studies established up-regulation of BRD9 in hepatocellular carcinomas[19] and its implication into SWi/SNF complexes, complexes that are involved in numerous types of cancers.[20]

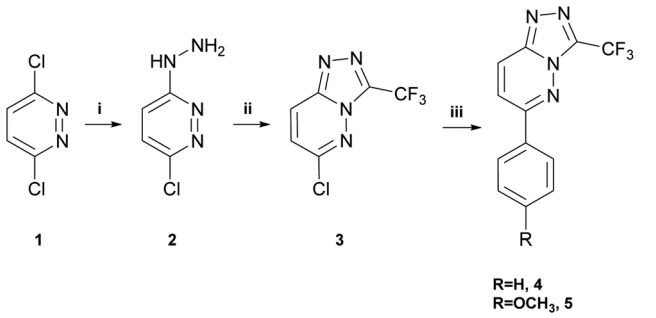
Chemistry

Bromosporine[16] (Fig. 1c) was used as the starting point for the design of a new series of molecules. Two trifluoromethyl analogues, as well as sulfonamide group elaboration of bromosporine were developed to explore either enhanced binding or improved selectivity towards BRD9's bromodomain.

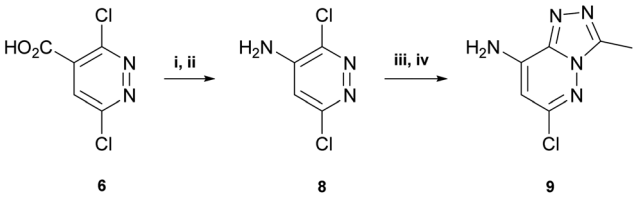
The preparation of the trifluoromethyl analogues is depicted in Scheme 1. 3,6-Dichloropyrazine **1** was first converted to its mono hydrazine derivative **2** and subsequent ring closing with trifluoroacetic acid afforded the triazolopyrazine scaffold **3**. Finally, a Suzuki coupling afforded compounds **4** and **5** in moderate yields using batch microwave chemistry.

The preparation of the second series of analogues based on the amino triazolopyrazine core **9** proved to be more challenging and the application of new flow chemistry technologies was considered as an alternative approach. We chose a Curtius rearrangement as the most expeditious route for the conversion of commercially-available 3,6-dichloropyridazine-4-carboxylic acid **6** to amine intermediate **8** (Scheme 2). The Curtius rearrangement requires heating of stoichiometric quantities of diphenyl phosphoryl azide (DPPA), a hazardous reagent, and results in the release of a large volume of nitrogen gas upon conversion to the product.

The advantages associated with performing large scale Curtius rearrangement in flow had been demonstrated previously by our group.[21] The need for large quantities of compound **9** led us to devise an automated synthesis approach for the preparation of the key building block **7** (Fig. 2). By using a flow reactor, we could perform both the generation of the intermediate acyl azide and the subsequent rearrangement in a continuous



Scheme 1 Synthesis of compounds **4** and **5** in the trifluoromethyl series. *Reagents and conditions*: (i) hydrazine monohydrate, ethanol, microwave heating, 100 °C, 60 min, quant. yield; (ii) trifluoroacetic acid, microwave heating, 110 °C, 70 min, 85%; (iii) phenylboronic acid or *para*-methoxyphenylboronic acid, $Pd(PPh_3)_4$ (3 mol%), sat. Na_2CO_3, ethanol–toluene 3 : 1, microwave heating, 120 °C, 120 min, 24–44%.



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Scheme 2 Synthesis of amino triazolopyrazine **9**. *Reagents and conditions*: (i) (flow conditions) DPPA, *t*-BuOH, triethylamine, toluene–acetonitrile 7 : 3, 120 °C, 140 min, 39%; (ii) HCl (4 M in dioxane), dichloromethane, quant.; (iii) hydrazine monohydrate, water, microwave heating, 100 °C, 3 h, 75%; (iv) glacial acetic acid, microwave heating, 120 °C, 5 h, 79%.

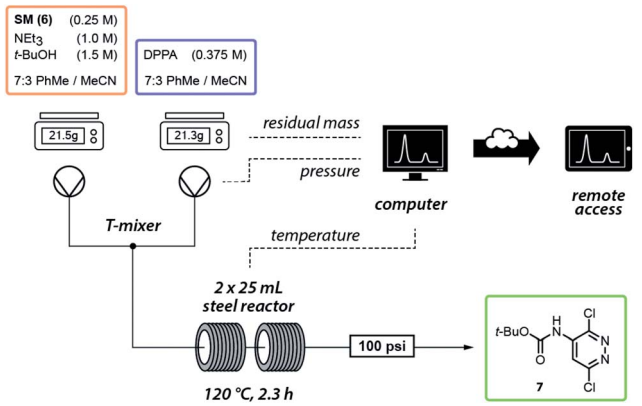


Fig. 2 Automated flow set-up for large scale Curtius rearrangement (40 mmol scale). A remote monitoring protocol allowed data from the reactor and the balances to be combined into a single display, which could be observed remotely over the internet.

sequence. This avoids any requirement to isolate large quantities of unstable acyl azide intermediate. Furthermore, only a small amount of the DPPA reagent is heated at any one time, allowing a large amount of material to be processed more safely than the corresponding batch procedure.[21,22] The use of an in-line pressure regulator controls the gas produced during the reaction.

A possible disadvantage associated with the flow method is its serial nature which can lead to long processing times for large scale operations. In this example, 40 mmol of product can be processed over 22 hours. The use of remote monitoring devices[23] allows us to observe reaction parameters such as temperature and pressure to ensure overall safety of the process. Connected digital balances could also be monitored to confirm accurate flow rates and consistent stoichiometric ratios over the extended reaction time. The subsequent Boc protecting group deprotection and triazole ring formation afforded over 1 g of **9**.

The required boronic ester partners **11–14** (Scheme 3) were obtained from the commercially available boronic acid **10** which was first converted to its corresponding pinacol ester and then reacted with aryl sulfonyl chlorides to afford intermediates **11–14**.

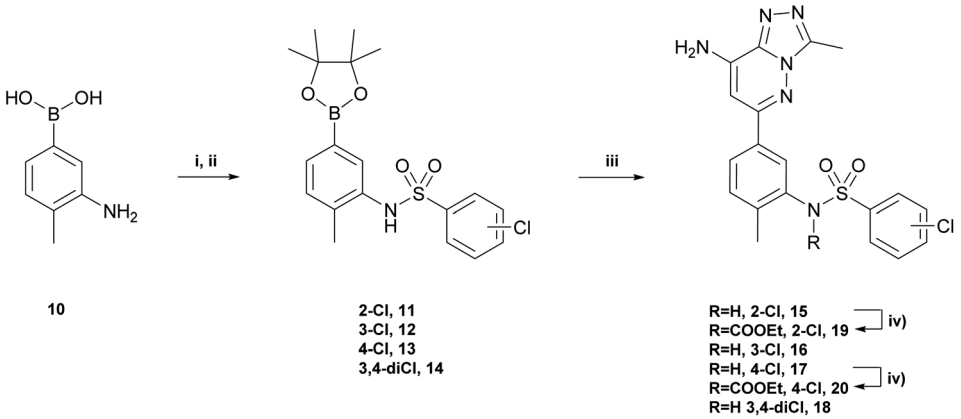
The triazolopyridazine core **9** proved to be a challenging partner for cross coupling reactions, possibly due to initial poisoning of the palladium catalyst by the nitrogen-containing heterocycles. Monodentate phosphine ligands for palladium, such as electron rich and bulky 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (XPhos), which is considered to be an effective ligand when dealing with heterocyclic substrates, were found to be unstable under the reaction conditions and significant palladium black deposition was observed. Bidentate phosphine ligands such as 1,1′-bis(diphenylphosphino)ferrocene (dppf) proved to be more stable under the reaction conditions, and extensive ligand screening identified the highly electron-rich and bulky 1,1′-bis(di-*tert*-butylphosphino)ferrocene (dtbpf) as the most promising ligand for palladium. This coupling then proceeded with a reasonable conversion and yield, and with selectivity for the heterocyclic chlorine target (**15–18**). It is worth mentioning however that our attempts to optimise the Suzuki coupling using an automated flow platform were not fruitful. By their very nature, on a small scale, meso-flow reactions often employ more reagents and solvents than corresponding batch processes.[24] Furthermore, the sensitivity of the reaction to oxygen and the low solubility of some of the reagents makes the reaction challenging, and is therefore better suited to batch processing using microwave irradiation.

Purification of the products by preparative HPLC furnished sulfonamides **15–18**. Based upon the structure of bromosporine, attempts were made to convert the free amine groups to their ethyl carbamate derivatives. Interestingly, the pendant amine of the heterocyclic core seems to be strongly deactivated following installation of the sulfonamide moiety, rendering its further decoration very difficult. As a result, only the analogues **19** and **20** were prepared, purified and evaluated as BRD9 binders (*vide infra*).

Evaluation of binding affinity for the BRD9 bromodomain using FAC-MS

FAC is a biophysical method which requires the injection of large plugs of analyte(s) into a column containing the immobilised target of interest, hence reaching saturation of the target (indicated by a plateau in ion count for every injection). Consequently, we needed to firstly modify the FAC apparatus to accommodate the small quantities of overexpressed BRD9 bromodomain. In order to achieve this, biotinylated BRD9 bromodomain was over-expressed and purified according to previously published procedures.[13] This target protein was then immobilised on streptavidin coated beads which resulted in a custom made column (15 μL column, Fig. 3a).

The packed column was validated by injecting bromosporine and one other known binder[16] (compound **21**, Fig. 3b) at different concentrations (for details, see Experimental section), giving access to the loading of functional BRD9 bromodomain, which was determined to be approximately **2** nmol. Furthermore, in all cases (*i.e.* bromosporine and its analogue **21**, both in Phosphate Buffer Saline – PBS – and 100 mM ammonium acetate), affinity constants compared well with literature IC_{50} data,[16] with the same order of magnitude and the same trend. Non-specific interactions were ruled out by injecting a void



Scheme 3 Synthesis of compounds **15–20**. *Reagents and conditions*: (i) pinacol, acetonitrile, 25 °C, 18 h; (ii) aryl sulfonyl chloride, pyridine, ethanol, 25 °C, 18 h, 63–77% over two steps; (iii) triazolopyridazine core **9**, potassium phosphate, [1,1′-bis(di-*tert*-butylphosphino)ferrocene] dichloro palladium(II), *n*-butanol–water 7 : 3, 1–35%; (iv) ethyl chloroformate, triethylamine, tetrahydrofuran, 25 °C, 5 min, 95%.

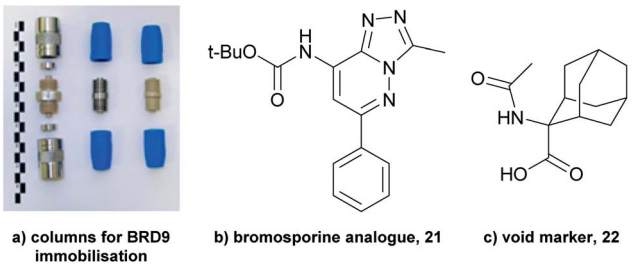


Fig. 3 BRD9 immobilisation and assessment of the resulting column: (a) commercially available (left, Kinesis) and custom made columns (center and right); (b) structure of the bromosporine analogue **21** and void marker **22**.[25]

marker (Fig. 3c) and bromosporine into a blank column; in these conditions, bromosporine did not show any significant retention. The protein loading on the column implies that low micromolar concentrations need to be accurately detected in order to provide reliable information. The system was further coupled to a CMS, which provided better sensitivity than UV detection (see ESI†), as well as detection of compounds that are not UV active (such as the void marker **22**). Although mass spectroscopy is not compatible with non-volatile buffers such as PBS, we were pleased to find that the BRD9 bromodomain was stable and functional in an ammonium acetate buffer. In order to compare the affinity of analogues **4**, **5**, and **15–20**, they were injected separately into the FAC-MS system which contained the BRD9 bromodomain column. The results obtained for the affinity screen are shown in Fig. 4.

We have previously reported that the correlation between the calculated affinity of compounds for their target (as K_d determined by several injections at different concentrations) and the retention time measured on single injections (one injection at one concentration),[1] using the principles of frontal affinity chromatography.[26] With single injections, we were able to rank quickly the compounds under study. Gratifyingly, the same

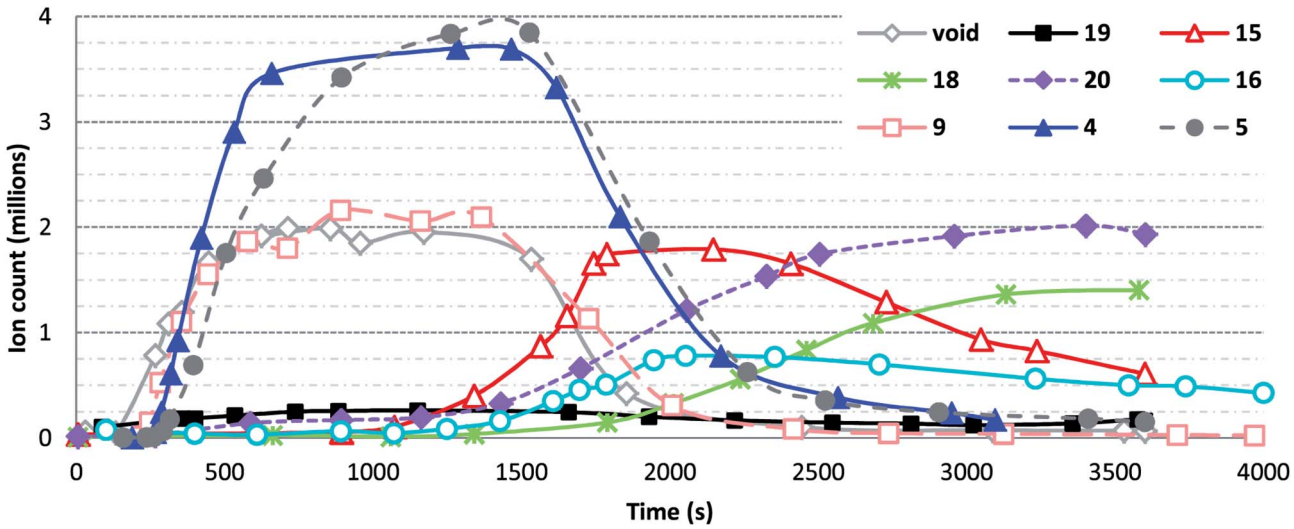


Fig. 4 Frontal affinity chromatography assay. Direct estimation of affinity towards the bromodomain of BRD9 protein by comparing retention times of void marker **22** and synthesised compounds **4**, **5**, **9**, **15**, **16** and **18–20** at the same concentration (4 μM).

Table 1 Retention times calculated from FAC-MS assay and thermal stabilisation of BRD9 bromodomain-containing protein

Compound	$V_{retention}$[a] (μL)	BRD9[b] ($\Delta T_{m,obs}$)
Void	160	n/a
4	212	−0.6
5	270	+0.6
15	784	+2.4
18	1167	+4.3
20	947	+2.7
Bromosporine	>3600[c]	+8.8

[a] Elution volume for which 50% of the ion count is attained. The retention volume was determined using a custom-made Excel file. [b] Difference in temperature of denaturation of the protein with and without the ligand, data in °C. [c] At a similar concentration (4 μM), bromosporine did not elute during the 120 min run; it was gradually eluted over a long period and no plateau was detected. An accurate breakthrough volume can be calculated for bromosporine using a more concentrated sample.

trend was confirmed by a separate thermal shift assay (Table 1), in accordance with a correlation previously reported between thermal shift data and affinity constants.[13]

The results showed that the BRD9 bromodomain column was of appropriate dimensions for rapid screening purposes. Furthermore, the difference of retention volumes obtained is significant enough to allow for the accurate distinction between low to medium (typically hundreds of micromolar) affinity compounds, making this approach of potential value in fragment-based drug discovery. Only **2** nmol of protein target (*i.e.* around 35 μg of recombinant biotinylated BRD9 bromodomain) were sufficient to carry out assays.

During a period of six months, no obvious loss of binding capacity for bromosporine was noticed (see Experimental section). This means that unlike conventional bioassays, evaluating a larger collection of compounds does not require a larger amount of protein which can be very beneficial and consequently may help overcome the disconnection between chemistry and biology. Consistent data were achieved and cross-validated by a widely used biophysical technique, namely a thermal shift assay.

A thermal shift assay against eight other proteins that are spread across the bromodomain phylogenetic tree[8] was also carried out (see ESI†). Compounds **4**, **5** and **20** displayed no noteworthy stabilisation of any of the bromodomains tested. Compound **15** on the other hand showed a mild selectivity for the first BRD4 bromodomain, while the most potent compound of the series, compound 18, exhibited stabilisation of both BRD4 bromodomain and the transcription factor EP300, hence did not display any selectivity for the BRD9 bromodomain.

Conclusions

In conclusion, a combination of flow and microwave chemistry methods quickly delivered eight analogues of bromosporine that were subsequently evaluated for their affinity for bromodomain proteins. In particular, the implementation of an automated Curtius rearrangement reaction facilitated the processing of a large amount of material in a safer manner than the corresponding batch process. The remote monitoring of this hazardous process ensured its safety. However, optimising a challenging cross coupling step using this automated platform was not successful; this is an area of ongoing research within our and other groups.[27] A simple, robust and reliable screening method using frontal affinity chromatography coupled with mass spectrometry afforded rapid binding information towards the BRD9 bromodomain that was additionally confirmed by a thermal shift assay.

Future work will include the preparation of an array of bromodomain containing columns to allow for rapid screening for affinity and selectivity. The development of more selective inhibitors and the exploration in more detail of fragment-based discovery of bromodomain modulators, both employing our flow assisted synthesis capabilities combined with the FAC-MS assay set-up, is on-going in our laboratory.

Experimental section

Chemistry

Synthetic procedures and characterisation can be found in the ESI.†

Frontal affinity chromatography

General considerations. Ammonium acetate was bought from Breckland Scientific. Streptavidin coated polyacrylate beads (Streptavidin Plus UltraLink Resin) were purchased from Pierce. Phosphate buffer saline (PBS) and formic acid were bought from Aldrich, HPLC grade methanol from Fischer Scientific. 100 mM ammonium acetate buffer was prepared with HPLC grade water (Rathburn) and was sterilised by filtration (Millipore, 0.22 μM) prior to use. DMSO (Alfa Aesar) was used without any further purification. The 15 μL guard column (1 mm × 2 cm) was purchased from Kinesis. FAC assays were run on a modified Agilent 1100 HPLC system with a 100 μL injection syringe and 100 mM ammonium acetate was used as the eluent. The temperature of the thermostatic oven was set at 20 °C. An Expression CMS single quadrupole mass spectrometer (Advion) was connected *via* a T-piece to the HPLC output and to a pump which delivered solution of 0.1% formic acid in methanol (make-up buffer). Stock solutions of compounds were prepared at a concentration of 50 mM in DMSO and stored at −20 °C.

Methods. The HPLC system was set to inject 600 μL of 4 μM of analyte at 30 μL min^{-1} for 120 min. The make-up buffer (0.1% formic acid in methanol) pump was set at a flow rate of 200 μL min^{-1}. Two wavelengths were simultaneously monitored at 220 nm (DMSO) and 254 nm (analyte) using a Diode Array UV Detector, and the mass detection on the Expression CMS was used in positive mode with Selective Ion Mode (SIM, span 1, dwell time 500 ms).

Preparation and assessment of the BRD9 bromodomain column. The 15 μL column was packed with streptavidin beads, installed onto the HPLC stack and washed for 30 min at 50 μL min^{-1} with PBS buffer. 100 μL of a solution of biotinylated bromodomain of BRD9 protein in PBS, which was

overexpressed according to previously published procedures,[13] was then injected three times through the column, at a flow rate of 50 μL min^{-1}. The column was subsequently evaluated with bromosporine. In practice, from a 50 mM stock solution of bromosporine in DMSO were prepared solutions at concentrations of 15, 7.5, 3.75, and 1.875 μM, both in PBS and in 100 mM ammonium acetate buffer. Each of these solutions was then infused in duplicate following the HPLC method described in the previous section, and the amount of active loaded protein (B_t) and affinity constant (K_d) values calculated as explained previously.[1] In these conditions, K_d and B_t were respectively found to be 1.48 ± 0.42 μM and 2.07 ± 0.23 nmol, satisfyingly both in PBS and in 100 mM ammonium acetate buffer. The same method was employed with the analogue of bromosporine **21** and gave similar B_t and a K_d of 8.35 ± 1.25 μM. In order to show the reproducibility of this assay, the same experiment was performed after six months on the same column, and no change in K_d or B_t was detected within experimental error. Additionally, during the six-month period in which the column was used recurrently, the breakthrough volume for a 50 μM sample of bromosporine was found to be constant at 324 ± 8 μL, validating the stability of the immobilised bromodomain protein on the column as well as the reproducibility of the technique. When not in use, the column was stored at 4 °C in a buffer solution (either PBS or 100 mM ammonium acetate) containing 0.1% sodium azide.

Thermal shift assay

Thermal melting experiments were carried out using a Mx3005p Real Time PCR machine (Stratagene) as previously described.[28]

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