Controlling Intramolecular Interactions in the Design of Selective, High-Affinity, Ligands for the CREBBP Bromodomain

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ABSTRACT

CREBBP (CBP or KAT3A) and its paralogue P300 (also KAT3B) are lysine acetyltransferases (KATs) that are essential for human development. They each comprise ten domains through which they interact with over 400 proteins, making them important transcriptional co-activators, and key nodes in the human protein-protein interactome. The bromodomain of CREBBP and P300 enables binding of acetylated lysine residues from histones, and a number of other important proteins, including p53, p73, E2F and GATA1. Here we report work to develop a high affinity, small molecule, ligand for the CREBBP and P300 bromodomains [(-)-OXFBD05] that shows >100-fold selectivity over the BET bromodomains. Key to the development of (-)-OXFBD05 were fundamental studies on molecular conformation in solution and when bound to the CREBBP bromodomain. In particular, the effect of an intramolecular hydrogen bond on solution state conformation, and use of an amide bioisostere, enabled the development of (-)-OXFBD05. Initial cellular studies using this ligand demonstrate that inhibition of the CREBBP/P300 bromodomain in HCT116 colon cancer cells results in lowered levels of c-Myc, and a modest but repeatable reduction in H3K18 acetylation. In hypoxia (<0.1% O₂), inhibition of the CREBBP/P300 bromodomain results in enhanced stabilization of HIF-1α. This presents an opportunity for modulating proteins that are affected by HIF-1α levels, including ACE2, which mediates SARS-CoV-2 infection of human cells.
INTRODUCTION

CREBBP (also CBP or KAT3A) and its parologue P300 (also KAT3B) are lysine acetyltransferases (KATs)\textsuperscript{1,2} that are essential for normal human development. Somatic mutations in CREBBP and P300 are associated with a range of cancers\textsuperscript{3} germline CREBBP mutations are linked with Rubinstein-Taybi syndrome (RTS).\textsuperscript{4} This syndrome is characterized by growth impairment, learning difficulties, and distinctive facial and skeletal anomalies.\textsuperscript{5} RTS patients also have an increased likelihood of developing some forms of cancer.\textsuperscript{3} That germline mutations in CREBBP, but rarely those in P300, result in RTS, demonstrates the non-redundancy of these two proteins, an observation that is supported by experiments on embryo development in mice.\textsuperscript{6}

CREBBP and P300 comprise ten domains each: NRID; TAZ1; KIX; bromodomain; RING; PHD; KAT; ZZ; TAZ2 and IBiD (Figure 1), through which they interact with over 400 different proteins. These interactions make them important transcriptional co-activators, and key nodes in the human protein-protein interactome.\textsuperscript{5} CREBBP and P300 are both capable of acetylating lysine residues on all four histones, although they show differing selectivities for which lysine residues they target.\textsuperscript{7} While the histone-targeted KAT activity of CREBBP/P300 has been heavily studied, they also acetylate a number of important non-histone proteins including p53, p73, E2F and GATA1,\textsuperscript{3} meaning that CREBBP/P300 are involved in multiple signaling pathways. Despite the advances made in our understanding of CREBBP/P300 KAT function, less is known about the specific role of the other protein domains.\textsuperscript{8} This has prompted work to make small molecule probes for these domains, including the KIX,\textsuperscript{9} TAZ1,\textsuperscript{10} NRID,\textsuperscript{11} and bromodomain,\textsuperscript{12-22} in addition to
the KAT domain.\textsuperscript{23} These molecules are starting to allow dissection of the specific role of a given domain within the context of the whole protein function.

**Figure 1.** A. The domain structure of CREBBP and P300, which comprise ten regions: NRID (N-terminal nuclear receptor interaction domain); TAZ1 (transcription adaptor zinc finger 1); KIX (kinase inducible); BRD (bromodomain); RING (really interesting new gene); KAT/HAT (lysine/histone acetyltransferase; ZZ (zinc finger); TAZ2 (transcription adaptor zinc finger 1); IBiD (interferon binding domain).\textsuperscript{8,24} B. An X-ray crystal structure of the BRD, RING, PHD and KAT domains of P300 bound to Lys-CoA (carbon =
aquamarine) obtained by Delvecchio et al.\textsuperscript{24} overlaid with an X-ray crystal structure of compound 1 (carbon = yellow) bound to the CREBBP bromodomain (PDB code 4NYX).\textsuperscript{12}

Over the last decade bromodomains have emerged as exciting targets in medicinal chemistry.\textsuperscript{25-29} While work has focused on the development of ligands for the BET bromodomains\textsuperscript{30} as a result of their role in a number of cancers,\textsuperscript{31} more recent work has seen the development of high affinity ligands for non-BET bromodomains.\textsuperscript{32-35}

Building on pioneering work by Zhou,\textsuperscript{36} who reported the first ligands for the CREBBP bromodomain, we reported the first high affinity ligands for the CREBBP/P300 bromodomain.\textsuperscript{12} This study identified the key interactions required for binding to the CREBBP bromodomain and these findings have subsequently underpinned the development of a number of other CREBBP bromodomain ligands.\textsuperscript{37} The use of some of these ligands has demonstrated that inhibition of the CREBBP/P300 bromodomain prevents growth of castration-resistant prostate cancer.\textsuperscript{38} Here we report ligand development and optimization building on our initial series of compounds, resulting in a high affinity (ITC $K_d$ = 102 ± 10 nM) CREBBP bromodomain ligand, ($-$)-OXFBD05 (2). This compound binds selectively to the CREBBP and P300 bromodomains, is >100-fold selective over BRD4(1) and shows no binding to a phylogenetically diverse panel of 10 bromodomains at a concentration of 1 μM. The enantiomeric companion compound, (+)-OXFBD05 (3), shows no binding to the CREBBP and P300 bromodomains and does not bind to the same panel of 10 bromodomains. Studies in HCT116 colon cancer cells demonstrate that inhibition of the CREBBP/P300 bromodomain results in downregulation of c-Myc, which is consistent with the hypothesis that ($-$)-OXFBD05 (2) is selectively
inhibiting the CREBBP/P300 bromodomains in this cell line. A modest but repeatable reduction in H3K18 acetylation is observed, demonstrating that the bromodomain plays a role in the KAT function of CREBBP/P300. In hypoxia, stabilization of HIF-1α above the level observed in hypoxia alone was observed. In contrast, the inactive enantiomer, (+)-OXFBD05 (3), shows none of these effects in the same cell line.
RESULTS AND DISCUSSION

Compound 1 proved to be a powerful tool for determining the structural requirements for small molecule binding to the CREBBP bromodomain, however, it retained low micromolar affinity for the BET bromodomains. The strong BET phenotype means that ligands for other bromodomains need to be highly selectivity for their target bromodomain over the BET family to be useful in cellular studies. Therefore, we wanted to develop compounds that show at least 100-fold selectivity for CREBBP/P300 over other bromodomain targets, while retaining high CREBBP bromodomain affinity. To this end, we investigated the SAR of 1. Compound 1 comprises three key sections, the KAc mimic, a tetrahydroquinoline (THQ) group that interacts with R1173 and the hydrophobic leucine, proline, phenylalanine (LPF) shelf, and a linker that joins these two motifs (Figure 2). The KAc mimic and the THQ moiety form the key interactions with the CREBBP/P300 bromodomain, while the linker adopts a conformation that enable these interactions. To optimize CREBBP/P300 bromodomain affinity and selectivity we have explored the limits of SAR in each of these regions (Figure 2). These investigations are discussed in detail below.
**Figure 2.**

A. The three regions of SAR explored in compounds 1 (R = OMe) and 4 (R = H). B. An X-ray crystal structure of compound 1 (carbon = yellow) bound to the CREBBP bromodomain (PDB code 4NYX). The dihydroquinoxalinone headgroup binds to the KAc pocket forming hydrogen bonding interactions with N1168. C. The electron-rich 7-(methoxy)tetrahydroquinoline group of 1 forms cation-π interactions with the positively charged R1173.12

**INVESTIGATING THE CATION-π INTERACTION**

A notable characteristic of the CREBBP bromodomain is the basic rim of the KAc-binding pocket, which includes R1173.36 Only three other bromodomains [EP300, PHIP(2) and BWRD3] share this feature, meaning it is a potential source of ligand selectivity. A key feature of compounds 1 and 4 binding to the CREBBP bromodomain is the interaction of the THQ group with R1173, which was initially observed by X-ray crystallography (PDB codes 4NYW, R = H; and 4NYX, R = OMe).12 We saw a change in CREBBP bromodomain IC₅₀ value from 4 (IC₅₀=758 nM) to 1 (IC₅₀=323 nM), indicating that increasing electron density in this region of the ligand might enhance its affinity for the bromodomain. Although not explicitly captured in the molecular mechanics force-fields, MD simulations
indicated that the cation-π between 1 and the CREBBP bromodomain can form spontaneously in water. DFT calculations estimate that this interaction contributes 3.2-4.7 kcal mol⁻¹ to the CREBBP bromodomain affinity of 1.¹²

We postulated that electron-rich THQ analogues would result in a strong cation-π interaction, and a computational model was developed to calculate the electrostatic surface potential (ESP) of THQ derivatives (1, 4-14, Figure 3).³⁷ We had previously established that substitution on the 7-position of the THQ ring is most beneficial for affinity, therefore the SAR explored on this ring focused on modifying this position.¹² The methoxy-substituted THQ derivative found in 1 was predicted to be the most mesomerically electron donating, with methoxy-indole 7 ESP expected to be comparable. We also studied the effect of removing the saturated ring system. Compounds 11 and 12 are the most electron-rich aromatic derivatives in this series (Figure 3). To explore the effects of a completely removing the aromatic system, the THQ ring was replaced with a methoxy substituent (15, Table S1). As the THQ moiety in the protein-ligand co-crystal structure of 1 (Figure 1) is partially solvent exposed, we prepared bulky tetrahydropyran derivatives 16-18, with the aim of improving selectivity over the BET bromodomains. These compounds were synthesized as shown in Scheme 1, and a full description of the synthesis is given in the SI.
Figure 3. 2D generated ESP isosurfaces of THQ analogues (1, 4-14) with differing substituents on the 7-position. Red indicates electron-dense regions and blue indicates electron-deficient regions. An additive model of two ESP values computed at 3.5 Å above the center of the aromatic ring of the ligand sidechain, and 3.5 Å above the center of the substituent on that aromatic ring, is shown below the surfaces.\textsuperscript{37}
**Scheme 1.** Synthesis of compounds probing the SAR of R1173 cation-π interaction.

(A) Synthesis of the dihydroquinoxalinone 23. **Reagents and conditions:** (a) BnBr, K$_2$CO$_3$, DMF, 60 °C, 3 h, 93%; (b) d-Alanine methyl ester hydrochloride, Cs$_2$CO$_3$, toluene, 85 °C, 18 h, 90%; (c) Zn, NH$_4$Cl, DMF, rt, 25 h, 82%; (d) H$_2$, 10% Pd/C, EtOAc, rt, 17 h, 99%.

(B) Synthesis of the tetrahydroquinoline-based compounds 1 and 4-6. **Reagents and conditions:** (e) LiAlH$_4$, THF, rt, 97%; (f) 25-27, K$_2$CO$_3$, 3-bromopropyl-N-phthalimide, DMF, 85 °C, 20 h; 20–60%; (g) 27, K$_2$CO$_3$, 3-bromopropyl-N-phthalimide, DMF, 85 °C, 20 h; 20%, then H$_2$, 10% Pd/C, MeOH, CH$_2$Cl$_2$, rt, 3 h, 69%, then AcCl, NEt$_3$, CH$_2$Cl$_2$, 0 °C to rt, 1 h, 74%; (h) Hydrazine monohydrate, MeOH, CH$_2$Cl$_2$, 70 °C, 2 h, 32–96%; (i)
23, PyBOP, NEt₃, DMF, rt, 46–77%; (C) Synthesis of indoles 8 and 7. **Reagents and conditions**: (j) K₂CO₃, 3-bromopropyl-N-phthalimide, DMF, 85 °C, 20 h; 69%; (k) MnO₂, CH₂Cl₂, 40 °C, 16 h, 78%; (m) Hydrazine monohydrate, MeOH, CH₂Cl₂, 70 °C, 2 h, 53–67%; (n) 23, PyBOP, NEt₃, DMF, rt, 73–89%; (D) Synthesis of (phenyl)amino derivatives 9-14 and 16-18. **Reagents and conditions**: (p) K₂CO₃, 3-bromopropyl-N-phthalimide, DMF, 85 °C, 20 h; 47–58%; (q) 46, hydrazine monohydrate, MeOH, CH₂Cl₂, 70 °C, 2 h, 96%; (r) 46-49, Mel, K₂CO₃, DMF, 85 °C, 2–15 h, 29–74%, then hydrazine monohydrate, MeOH, CH₂Cl₂, 70 °C, 2 h, 50–89%; (s) 50, Mel, K₂CO₃, DMF, 85 °C, 5 h, 71%, then mCPBA, CH₂Cl₂, rt, 3 h, then B₂(OH)₄, MeOH, rt, 14 h, 75% over two steps, then hydrazine monohydrate, MeOH, CH₂Cl₂, 70 °C, 2 h, 71%; (t) 47-49, 4-(iodomethyl)tetrahydro-2H-pyran, K₂CO₃, DMF, 85 °C, 15–20 h, 11–58%, then hydrazine monohydrate, MeOH, CH₂Cl₂, 70 °C, 2 h, 51–97%; (u) 23, PyBOP, NEt₃, DMF, rt, 38–76%.

The CREBBP bromodomain affinity of this series of compounds was initially investigated using an AlphaScreen assay (Table S1). The compounds that showed higher affinity in this assay were re-evaluated using ITC (Table 1). Plotting the calculated ESP against the measured Kₐ value reveals a good correlation (Figure 4; r² = 0.77) between these two values, demonstrating the importance of the interaction with R1173; complete removal of the THQ moiety abrogates all CREBBP bromodomain affinity. Generally, compounds that can form stronger electrostatic interactions with R1173 show higher affinity for the CREBBP bromodomain. The reduction in affinity observed for monocyclic systems suggests that some hydrophobic interactions with the LPF shelf region also contribute to ligand affinity. This analysis confirms that, of the analogues assessed, compound 1 shows
the highest affinity for the CREBBP bromodomain, and that there is little room for improving this affinity by optimizing this region of the ligand. Therefore, the 7-methoxy THQ motif was retained in our subsequent studies.
Table 1. CREBBP and BRD4(1) \( K_d \) values (ITC), \( pK_d \) values, and CREBBP vs BRD4(1) selectivity for compounds 1, 4, 6, 7, 10-14, and 16-18. ITC data: Figures S12 and S13.

![Diagram](HN NH HN O O)

| Compound number | Substituent | CREBBP \( K_d \) (μM) (ITC) | CREBBP \( pK_d \) | BRD4(1) \( K_d \) (μM) (ITC) | BRD4(1) \( pK_d \) | CREBBP/BRD4(1) selectivity |
|-----------------|-------------|-----------------------------|-----------------|-----------------------------|-----------------|-----------------------------|
| 1               |             | 0.353 ± 0.06 (0.390\(^a\)) | 6.5             | 1.01 ± 0.30 (1.40\(^a\))   | 6.0             | 2.9                         |
| 4               |             | 1.53 ± 0.28\(^b\)          | 5.7             | n.t.                        | n.t.            | -                           |
| 6               |             | 1.21 ± 0.33                | 5.9             | 1.57 ± 0.51                 | 5.8             | 1.3                         |
| 7               |             | 0.720 ± 0.14               | 6.1             | 2.90 ± 1.12                 | 5.5             | 4.0                         |
| 10              |             | 1.43 ± 0.57                | 5.8             | n.t.                        | n.t.            | -                           |
| 11              |             | 0.601 ± 0.08               | 6.2             | 2.47 ± 0.86                 | 5.6             | 4.1                         |
| 12              |             | 1.19 ± 0.26                | 5.9             | n.t.                        | n.t.            | -                           |
| 13              |             | 1.48 ± 0.33                | 5.8             | n.t.                        | n.t.            | -                           |
| 14              |             | 1.91 ± 0.54                | 5.7             | n.t.                        | n.t.            | -                           |
| 16              |             | 0.734 ± 0.19               | 6.1             | 1.60 ± 0.39                 | 5.8             | 2.2                         |
| 17              |             | 1.93 ± 1.00                | 5.7             | n.t.                        | n.t.            | -                           |
| 18              |             | 2.84 ± 1.23                | 5.5             | n.t.                        | n.t.            | -                           |

\(^a\)Previously published data.\(^b\) n.t.: not tested. ± standard error of the fit unless stated. A heatmap representation is used; high affinity or selectivity are indicated by hot colors. \(^n=4 ± s.d. from the mean.\)
Figure 4. Linear regression of the electrostatic potential and the CREBBP affinity of 1, 6, 7, 11-13 shows a good correlation ($r^2 = 0.77$).
INVESTIGATING THE LINKER CONFORMATION

When bound to the CREBBP bromodomain, the linker of 4 adopts a twisted conformation, with the amide and THQ nitrogen atoms gauche (Figure 5A). We hypothesized that predisposing the linker to adopt this conformation in solution would result in a ligand with higher affinity and selectivity for the CREBBP bromodomain. One approach to achieve this might be through the fluorine gauche effect, which has been harnessed to manipulate the conformation of alkyl chains in biologically active molecules.\textsuperscript{39,40} Most relevant to our system, O'Hagan et al. showed that N-β-fluoroamides prefer to adopt a gauche conformation, with an energy difference of 1.8 kcal mol\textsuperscript{-1} over the anti-conformation.\textsuperscript{41} Based on this observation, we designed compound 60 (Scheme 2), with a geminal difluoro motif at the center of the linker. In the desired conformation, we predicted that at least one of the fluorine atoms would sit gauche to the amide, favoring this orientation. Compound 60 was synthesized as shown in Scheme 2, and a full description of the synthesis is given in the SI.

![Scheme 2](image)

**Scheme 2.** Synthesis of difluoropropyl-containing ligand 60. *Reagents and conditions:* (a) \textit{1H}-Benzotriazole, 37\% aq. CH\textsubscript{2}O, MeOH, Et\textsubscript{2}O, 50 °C, 4 h, 92\%; (b) TMSCl, Zn, rt, 10 min, then BrCF\textsubscript{2}CO\textsubscript{2}Me, rt, 10 min, then 61, 60 °C, 75 min, then LiAlH\textsubscript{4}, THF, rt, 1 h, 27\% over 2 steps; (c) MsCl, NEt\textsubscript{3}, CH\textsubscript{2}Cl\textsubscript{2}, rt, 3 h, 97\%, then NaN\textsubscript{3}, DMF, 110 °C, 16 h, 86\%; (d) H\textsubscript{2}, 10\% Pd/C, EtOAc, rt, 16 h, 97\%, then 23, PyBOP, NEt\textsubscript{3}, DMF, rt, 17 h, 68\%. 
Table 2. CREBBP $K_d$ values (ITC), $pK_d$ values, IC$_{50}$ values (AlphaScreen) and pIC$_{50}$ values for compounds 4 and 60. For ITC plots and binding isotherms see Figure S12.

| Compound number | R | CREBBP $K_d$ (μM) (ITC) | CREBBP $pK_d$ | CREBBP IC$_{50}$ (μM) (AlphaScreen) | CREBBP pIC$_{50}$ |
|-----------------|---|------------------------|-------------|----------------------------------|------------------|
| 4               | H | 1.53 ± 0.28$^a$        | 5.7         | 3.1 ± 0.30                       | 5.5              |
| 60              | F | 0.388 ± 0.01           | 6.4         | 1.1 ± 0.20                       | 6.0              |

$^a$n=4 ± s.d. from the mean. A heatmap representation is used; high affinity is indicated by hot colors. ± standard error of the fit.

Addition of the geminal difluoro motif led to an increase in affinity for the CREBBP bromodomain (60, Table 2). We analyzed the ITC binding signature plot to investigate whether this additional affinity results from manipulation of the alkyl chain conformation. The plot shows that the ΔG value for compound 4 binding to the CREBBP bromodomain results primarily from enthalpic interactions (Figure S1A). However, approximately half of the ΔG for binding of compound 60 results from the entropy-related $-T\Delta S$ term (Figure S1B). The entropic component can indicate conformational pre-organization of the binding ligand, as less ligand entropy is lost on binding to the protein. However, this term can also relate to hydrophobic interactions, with an increase in entropy resulting from the displacement of weakly bound water molecules from the protein surface.$^{42}$ We observed that the solubility of 60 is lower than that of 4, indicating increased lipophilicity (cLogP 4 = 3.63; cLogP 60 = 4.30), which might be responsible for the increase in CREBBP bromodomain affinity. However, the LLE of the two compounds is the same (Table S1),
suggesting that at least some of the affinity from 60 results from conformation pre-
organization.

An X-ray crystal structure of 60 bound to the CREBBP bromodomain (Figure 5C PDB
code 6YIK C2 space group, and S2 PDB code 6YIL P2_1 space group, carbon = blue)
shows that this molecule adopts the same conformation as 4 when bound to the protein
(Figure 5C and S5, PDB code 4NYX, carbon = yellow). Overlaying a small molecule X-
ray structure of 60 (CCDC code 1993652-3, carbon = orange) with the co-crystallized
structure of 4 (Figure 5C) shows that the geminal difluoro group does affect the unbound
conformation of the chain. However, perhaps unsurprisingly, the solid-state conformation
is not identical to that of the protein-bound molecule. This is illustrated by considering the
CH^{b_2}-CH^{c_2} bond (Figure 5A and B), where we can see that the amide and THQ groups
adopt a gauche conformation for both molecules, when bound to the protein. In the small
molecular X-ray crystal structure 60 the amide group sits gauche to both of the fluorine
atoms, resulting in an anti-relationship between the amide and the THQ ring. By
considering the CH^{a_2}-CH^{b_2} bond (Figure 5D and E) we can see that the alkyl chain and
the THQ group adopt an anti-conformation in both protein-bound molecules. However, in
the small molecule X-ray crystal structure of 60, the THQ and the alkyl chain are gauche.
From the protein X-ray crystal structure, it can also be seen that the geminal difluoro
group forms an hydrophobic interaction with P1110 (Figure 5C).
Figure 5. A comparison of the conformations adopted by compounds 4 and 60 bound to the CREBBP bromodomain with the conformation of 60 observed in a small molecule X-ray crystal structure. A. Considering the CH\textsubscript{b2}-CH\textsubscript{c2} bond shows that the amide and THQ groups of 19 adopt a gauche conformation when bound to the protein (yellow box). B. Considering the CF\textsubscript{2}-CH\textsubscript{c2} bond shows that the amide and THQ groups of 61 also adopt a gauche conformation when bound to the protein (blue box). In the small molecule X-ray crystal structure (orange box), the amide and THQ groups are anti. C. The X-ray crystal structure of 60 bound to the CREBBP bromodomain (PDB code 6YIK, carbon = blue, protein surface from this structure shown) overlaid with the small molecule X-ray crystal structure of 60 (CCDC code 1993652-3, carbon = orange) and the X-ray crystal structure of 4 bound to the CREBBP bromodomain (PDB code 4NYX, carbon = yellow). D. Considering the CH\textsubscript{a2}-CH\textsubscript{b2} bond of 4 shows that the alkyl chain and the THQ group adopt an anti-conformation in the protein-bound conformation. E. Considering the CH\textsubscript{a2}-CF\textsubscript{2} bond shows that the alkyl chain and the THQ group of 60 adopt an anti-conformation in the protein-bound conformation of (blue box), but are gauche in the small molecule X-ray crystal structure (orange box). Low temperature single crystal X-ray diffraction data for 60 were collected using a Rigaku Oxford SuperNova diffractometer. Raw frame data were reduced using CrysAlisPro and the structures were solved using ‘Superflip’\textsuperscript{43} before refinement with CRYSALIS\textsuperscript{44,45} as per the SI. Full refinement details are given in the Supporting Information (CIF); Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre (CCDC 1993652).

Overall, these data are consistent with the geminal difluoro group providing a degree of conformation pre-organization in compound 60, which might result in higher affinity for
the CREBBP bromodomain. However, the hydrophobic nature of this group likely also contributes to the increased affinity of this ligand for the CREBBP bromodomain. The hydrophobic nature of this molecule was evident in a protein X-ray crystal structure obtained with the P2₁ space group (PDB code 6YIL), in which we observed a 3:5 protein to ligand ratio, with the additional two ligands bound at the interface two bromodomains (Figure S2). Therefore, while this modification enhances the ligand-bromodomain affinity, its effect on compound solubility meant that it was not included in the next generation of ligands.

OPTIMISATION OF THE ACETYL-LYSINE MIMIC

During in vitro testing, we noticed that compound 1 underwent a small degree of oxidation when stored in DMSO. While stable for the duration of our assays, after 5 days stirring in deuterated DMSO at 50 °C we observed that 14% of the material was oxidized from the 3,4-dihydroquinoxalinone to the corresponding quinoxalinone (Scheme 3, Figure S3A). Compound 64 has reduced affinity for the CREBBP bromodomain (data not shown). We reasoned that expansion of the 6-membered 3,4-dihydroquinoxalinone ring to give a 4,5-dihydrobenzodiazepinone ring (65) would reduce the propensity for oxidation. From a synthetic perspective, this ring system is accessible as it can be constructed by switching the starting material from an α-amino acid to a β-amino acid.¹

¹ We note that the same KAc mimic was reported by Taylor et al. during the course of our studies.¹⁶
**Scheme 3.** Oxidation of the 3,4-dihydroquinoxalinone headgroup (1) and ring expansion to give the 4,5-dihydrobenzodiazepinone KAc mimic (65). *Reagents and conditions*: (a) DMSO, 50 °C, 5 days 14% conversion.

To establish whether the 4,5-dihydrobenzodiazepinone headgroup would be accommodated by the CREBBP KAc-binding pocket, we performed docking studies, using AutoDock Vina, followed by 120 ns molecular dynamic simulations (Figure S4). We initially validated this approach by replicating the binding pose observed in the X-ray crystal structure of 1 bound to the CREBBP bromodomain. We then replicated this procedure for the 4,5-dihydrobenzodiazepinone 65 using the apo-structure of the CREBBP bromodomain (PDB code 3P1C). The resulting predicted structure of 65 matched well with the X-ray crystal structure of 1 bound to the CREBBP bromodomain. The 4,5-dihydrobenzodiazepinone was clearly accommodated in the KAc binding pocket and hydrogen bonds were predicted with N1168. Additional hydrophobic interactions were predicted between the methylene unit and V1115 and I1122, which might result in increased affinity for the CREBBP bromodomain. In addition, the cation-π interaction between R1173 and THQ was predicted to occur by the MD simulations (Figure S4). These observations encouraged us to proceed with the synthesis of 65. We also synthesized the enantiomeric compound 66, and the isomer 67, in which the methyl group is moved from the 4-position to the 3-position of the 4,5-dihydrobenzodiazepinone ring.
(Scheme 4). The 4,5-dihydrobenzodiazepinone derivatives 65-67, were synthesized as shown in Scheme 4, and a full description of the synthesis is given in the SI.

Scheme 4. Synthesis of the 4,5-dihydrobenzodiazepinone-based compounds 65-67. (A)

Synthesis of β-amino acid ester derivatives 71, 72 and 75. Reagents and conditions: (a) N-benzyl-1-phenylethanamine, ⁶BuLi, THF, −78 °C, then 68, THF, −78 °C, 83%; (b) H₂, Pd(OH)₂/C, MeOH/H₂O, AcOH, 85%; (c) BnNH₂, DBU, 90 °C, 16 h, 51%. (B) Synthesis of the 4,5-dihydrobenzodiazepinone-based compounds 65-67. Reagents and conditions: (d) 71/72/75, Cs₂CO₃, toluene, 85 °C, 14 h, 83–97%; (e) TFA, CH₂Cl₂, rt, 2 h, then Fe, AcOH, 100 °C, 4 h, 65–89% over 2 steps; (f) H₂, 10% Pd/C, EtOAc, rt, 17 h, 99%, then 33, PyBOP, NEt₃, DMF, rt, 26–68% over 2 steps; (g) TFA, CH₂Cl₂, rt, 2 h, then Zn, NH₄Cl, DMF, rt, 15 h, then PyBOP, NEt₃, DMF, rt, 10% over 3 steps.
To establish whether compound 65 was less prone to oxidation than compound 1 it was also stirred in deuterated DMSO at 50 °C for 5 days. Pleasingly we saw no evidence of an oxidized product, indicating that 65 is stable under these conditions (Figure S3B).

When tested using ITC and AlphaScreen, compound 65 showed significant CREBBP bromodomain affinity, albeit slightly reduced compared to 1 (Table 3). In the 3,4-dihydroquinoxalinone series, the (S)-enantiomer showed an 8-fold reduction in affinity compared to the (R)-enantiomer.12 In the 4,5-dihydrobenzodiazepinone series, however, the (S)-enantiomer (66) showed no detectable affinity for the CREBBP bromodomain. This provides the opportunity to develop a useful inactive companion compound for use as a control in cellular experiments. The isomeric compound 67, which has the methyl group in the 3-position, also showed low CREBBP bromodomain affinity when tested as a racemate.
Table 3. CREBBP and BRD4(1) $K_d$ values (ITC), $pK_d$ values, CREBBP $IC_{50}$ values (AlphaScreen), $pIC_{50}$ values and CREBBP vs BRD4(1) selectivity for compounds 1 and 65-67. For ITC plots and binding isotherms see Figures S12 and S13.

![Chemical structure](image)

| Compound number | Head group | CREBBP $K_d$ (μM) (ITC) | CREBBP $pK_d$ | CREBBP $IC_{50}$ (μM) (Alpha Screen) | CREBBP $pIC_{50}$ | BRD4(1) $K_d$ (μM) (ITC) | BRD4(1) $pK_d$ | CREBBP / BRD4(1) selectivity |
|-----------------|------------|--------------------------|---------------|--------------------------------------|-------------------|--------------------------|---------------|-----------------------------|
| 1               |            | 0.353 ± 0.06 (0.390<sup>a</sup>) | 6.5           | 0.323<sup>a</sup>                   | 6.5               | 1.01 ± 0.30 (1.40<sup>a</sup>) | 6.0           | 2.9                         |
| 65              |            | 0.466 ± 0.03<sup>b</sup> | 6.3           | 1.40                                 | 5.9               | 2.82 ± 0.32<sup>b</sup> | 5.5           | 6.1                         |
| 66              | n.b.       | -                        | >50           | -                                    | n.t.              | -                        | -             | -                           |
| 67              | n.t.       | -                        | 10.2          | 5.0                                  | 2.90 ± 1.12       | 5.5           | 4.0                         |

<sup>a</sup>Previously published data.<sup>12</sup>{Rooney:2014gy} n.t.: not tested; n.b.: no binding. ± standard error of the fit unless stated. A heatmap representation is used; high affinity is indicated by hot colors. <sup>b</sup>n=2 ± s.d. from the mean.

We obtained an X-ray crystal structure of compound 65 bound to the CREBBP bromodomain (PDB code 6YIM, carbon = purple). Overlaying this structure with that of 1 bound to the CREBBP bromodomain (PDB code 4NYX, carbon = yellow) shows that these compounds have very similar binding modes (Figure 6). As with compound 1, the 4,5-dihydrobenzodiazepinone of 65 forms two hydrogen bonds with N1168. The amide carbonyl oxygen forms hydrogen bonds with a ZA-channel water molecule, and the THQ
ring interacts with R1173. The extra methylene unit of the 4,5-dihydrobenzodiazepinone is accommodated by movement of V1115, relative to the 4NYX structure, and this region of the pocket is more fully occupied by 65, compared to 1.

**Figure 6.** X-Ray crystal structure of 65 bound to the CREBBP bromodomain (PDB code 6YIM, carbon = purple, protein surface from this structure shown) overlaid with the X-ray crystal structure of 1 bound to the CREBBP bromodomain (PDB code 4NYX, carbon = yellow).\(^1\)\(^2\) A. The side orientation shows that the headgroups of each compound form the same hydrogen-bonding interactions with the bromodomain, and that the KAc-mimicking methyl and carbonyl groups of both molecules overlay very closely. B. The top orientation shows that the interaction with R1173 is present for both molecules. The kink in the 4,5-dihydrobenzodiazepinone ring of 65 (carbon = purple), which more fully occupies the KAc-binding pocket, is visible. See Figure S11A for ligand electron density map.
To gain an insight into the solution-state binding of compounds 1 and 65 to the CREBBP bromodomain, we employed protein-observed $^{19}$F (PrOF) NMR. To enable this approach, a fluorine-labeled CREBBP bromodomain was expressed in which the three naturally occurring tryptophan residues were replaced with 5-fluorotryptophan (Figure 7A). Unlike our previous reports of fluorinated bromodomains employed in PrOF NMR analysis, these tryptophans reside outside the binding site. Consequently, a change in their chemical environment can result from allosteric, or large scale, structural changes.

Figure 7. A. The X-ray crystal structure of 65 bound to the CREBBP bromodomain (PDB code 6YIM, carbon = purple, cartoon from this structure shown) overlaid with the X-ray crystal structure of 1 bound to the CREBBP bromodomain (PDB code 4NYX, carbon = yellow). The three tryptophan residues (W1151, W1158 and W1165) that were replaced by 5-fluorotryptophan are highlighted as green sticks. B. Partial $^{19}$F NMR spectra showing
the effect of adding 11.3, 22.6 or 45.2 µM of either 1 or 65 to the 5-fluorotryptophan-containing CREBBP bromodomain (45 µM).

The incorporated 5-fluorotryptophan residues give three distinct signals in the $^{19}$F NMR spectrum at $-123.5$ ppm, $-125.0$ ppm, and $-127.0$ ppm (Figure 7B). Upon the addition of compound 1 (11.3, 22.6 or 45.2 µM), the $^{19}$F NMR signal at $-123.5$ ppm shifted upfield to $-124.0$ ppm and the resonance at $-125.0$ ppm showed slight shift at the highest concentration. The resonance at $-127.0$ ppm was unaffected (Figure 7B). Addition of compound 65 (11.3, 22.6 or 45.2 µM) also resulted in a shift of 0.5 ppm for the resonance at $-123.5$ ppm. In addition, the other two resonances show small but discernible shifts, especially at the highest concentration. The observation of a shift of all three resonances upon addition of 65 supports the idea that a structural rearrangement is required to accommodate the larger 4,5-dihydrobenzodiazepinone ring in the KAc-binding pocket, resulting in more London dispersion forces between 65 and V1115 than for compound 1. Interestingly, when the experiment was repeated with I-CBP112$^{14}$ a similar shift was observed for the downfield resonance at $-123.5$ ppm, but in the opposite direction (Figure S5A). Addition of bromosporine,$^{49}$ which is a weaker binding ligand for the CREBBP bromodomain, did not affect the $^{19}$F chemical shifts (Figure S5B). While further investigation is needed, these data are consistent with the idea that 65 and I-CBP112 both bind to the bromodomain, but cause different structural rearrangements in the protein, which might result in different allosteric effects in the full-length protein (vide infra).
Although these results show that the 4,5-dihydrobenzodiazepinone motif was accommodated by the CREBBP bromodomain KAc-binding pocket, it did not result in the predicted increase in affinity, which was surprising. This observation led us to consider the effects of intramolecular hydrogens bonds in dictating the solution-state conformation of our CREBBP bromodomain ligands.

INVESTIGATING THE INTRAMOLECULAR HYDROGEN BOND

To investigate the presence of intramolecular hydrogen bonds in solution we have previously employed an $^1$H NMR-based approach. This method was recently used to investigate the presence of an intramolecular hydrogen bond in a series of BET bromodomain ligands. When changing the NMR solvent from CDCl$_3$ to D$_6$-DMSO the chemical shift of hydrogen atoms that are not involved in hydrogen bonds typically show a $\Delta_{\text{ppm}}$ CDCl$_3$ → D$_6$-DMSO = 2–4 ppm. This shift results from the solvent having the greatest effect on the environment of these atoms. Hydrogen atoms that are participating in an internal hydrogen bond generally show $\Delta_{\text{ppm}}$ CDCl$_3$ → D$_6$-DMSO <1 ppm. The environment of these atoms is mainly affected by the intramolecular interaction, and is less affected by the surrounding solvent. When combined with structural studies, this technique allows us to assess whether intramolecular hydrogen bonds formed in solution are also present when the ligand is bound to a protein.

During the development of compound 1, we showed that a resonance-assisted intramolecular hydrogen bond was present in the protein-bound X-ray crystal structure of this compound bound to the CREBBP bromodomain. Kuhn et al. have proposed that resonance-assisted hydrogen bonds can be observed in an X-ray crystal structure by
having $100 \degree \leq \alpha \leq 180 \degree$ ($\alpha$ angle is shown in Figure 8) and the donor-acceptor distance (d) $\leq 2.35$ Å (Figure 8). In addition, we propose that the O-C-N-H dihedral angle (Figure 8) should be sufficiently small to allow the $n\rightarrow\sigma^*_{N-H}$ overlap that is a key component of hydrogen bonding. These criteria are met by compound 1, indicating that this hydrogen bond exists in the X-ray crystal structure.
Figure 8. A. A resonance-assisted hydrogen bond is observed when 1 binds to the CREBBP bromodomain as α > 100 °, d < 2.3 Å and an O-C-N-H dihedral angle of −29.3 ° is observed (PDB code 4NYX, carbon = yellow). A Δ\_ppm CDCl\_3 → D\_6-DMSO of 0.08 ppm
for \( ^{a} \text{NH} \) indicates that the intramolecular hydrogen bond is present in solution. B. In a small molecule X-ray crystal structure of 65 a resonance-assisted hydrogen bond is observed as \( \alpha >100 ^\circ \), \( d <2.35 \text{ Å} \) and an O-C-N-H dihedral angle of \( -5.6 ^\circ \) is observed (CCDC code 1993652-3, carbon = pink). A \( \Delta_{\text{ppm}} \text{CDCl}_3 \rightarrow \text{D}_6\text{-DMSO} \) of \(-0.12 \text{ ppm} \) for \( ^{a} \text{NH} \) indicates that the intramolecular hydrogen bond is also present in solution. C. A resonance-assisted hydrogen bond is not observed when 65 binds to the CREBBP bromodomain as \( \alpha <100 ^\circ \), \( d >2.35 \text{ Å} \) and an O-C-N-H dihedral angle of \( -51.9 ^\circ \) is observed (PDB code 6YIM, carbon = purple). D. Overlay of the small molecule X-ray crystal structure (CCDC code 1993652-3, carbon = pink) with the conformation of 65 observed in the X-ray crystal structure of this compound bound to the CREBBP bromodomain CREBBP (PDB code 6YIM, carbon = purple). Low temperature single crystal X-ray diffraction data for 60 were collected using a Rigaku Oxford SuperNova diffractometer. Raw frame data were reduced using CrysAlisPro and the structures were solved using 'Superflip'\(^{43} \) before refinement with CRYSTALS\(^{44,45} \) as per the SI. Full refinement details are given in the Supporting Information (CIF); Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre (CCDC 1993652-3).

Using \(^1\text{H} \) NMR studies, we see that the chemical shifts for \( ^{b} \text{NH} \) and \( ^{c} \text{NH} \) (1, Figure 8A) have a \( \Delta_{\text{ppm}} \text{CDCl}_3 \rightarrow \text{D}_6\text{-DMSO} \) of 2.6 ppm, showing that, as expected, these atoms are not involved in hydrogen bonds. For \( ^{a} \text{NH} \), however, we see a \( \Delta_{\text{ppm}} \text{CDCl}_3 \rightarrow \text{D}_6\text{-DMSO} \) of only 0.08 ppm. These data are consistent with the presence of an intramolecular hydrogen bond in solution. This observation supports our hypothesis that the
intramolecular hydrogen bond helps to pre-organize the solution state conformation of 1 to one that is similar to the protein-bound conformation.

For compound 65, the chemical shifts for δNH and γNH (Figure 8) have a $\Delta_{\text{ppm}}$ CDCl$_3 \rightarrow$ D$_6$-DMSO of 2.4 and 2.2 ppm, respectively, indicating that, as expected, these atoms are not involved in hydrogen bonds. For aNH, we see a $\Delta_{\text{ppm}}$ CDCl$_3 \rightarrow$ D$_6$-DMSO of $-0.12$ ppm, indicating the presence of an intramolecular hydrogen bond in solution (Figure 8B and Figure S6). This hydrogen bond is also observed in a small molecular X-ray crystal structure of 65 as $\alpha > 100^\circ$, $d < 2.35$ Å and an O-C-N-H dihedral angle of $-5.6^\circ$ is observed (CCDC code 1993652-3, carbon = pink). However, analysis of the X-ray crystal structure of 65 bound to the CREBBP bromodomain shows an $\alpha < 100^\circ$, $d > 2.35$ Å, and a dihedral angle of $-51.9^\circ$ suggesting that there is no intramolecular hydrogen bond present when 65 is bound to the CREBBP bromodomain (Figure 8C). An overlay of the free and protein-bound X-ray crystal structures (Figure 8D) indicates that the 4,5-dihydrobenzodiazepinone ring has to undergo a ring flip to bind the CREBBP bromodomain. For this to occur, the intramolecular hydrogen bond has to be broken, explaining why 65 has lower affinity than 1 for the CREBBP bromodomain. If this hypothesis is correct, then replacement of the amide with a bioisostere that cannot hydrogen bond with aNH, but which retains the amide bond rigidity, should lead to a higher affinity CREBBP bromodomain ligand.
**INTRODUCING AMIDE BIOISOSTERES**

![Diagram of amide bioisostere replacements]

**Figure 9.** To investigate the effect of an intramolecular hydrogen bond on CREBBP bromodomain affinity we designed compounds in which the amide bond is replaced with an \((E)\)-alkene (2). For comparison we also designed compounds containing a \((Z)\)-alkene (82), an alkyne (83) and an alkane (84). We included the opposite enantiomer (3) of 2, which we predicted would show no CREBBP bromodomain activity, making it a useful companion control compound.

When considering a bioisosteric replacement for the amide bond we wanted to select a group that mimics the geometric constrains of a peptide bond, but which could not act as a hydrogen bond donor or acceptor. We predicted that such a group would maintain some pre-organization of the compound, but without the need to break the intramolecular hydrogen bond to allow protein binding, resulting in a higher affinity CREBBP bromodomain ligand. Based on these criteria we selected an \((E)\)-alkene as the simplest amide bioisostere (Figure 9).\(^{44,45,53}\) In addition, we decided to make the \((Z)\)-alkene (82), the alkyne (83), and the alkane (84) derivatives for comparison. We predicted that the
enantiomeric \((E)\)-alkene (3) would show no affinity for the CREBBP bromodomain. The 4,5-dihydrobenzodiazepinone derivatives 2, 3, 82, 83 and 84, were synthesized as shown in Scheme 5, and a full description of the synthesis is given in the SI.

**Scheme 5.** Synthesis of the 4,5-dihydrobenzodiazepinone-based compounds 2, 3, and 82-84. (A) Synthesis of 4,5-dihydrobenzodiazepinone headgroup. Reagents and conditions: (a) 71/72, DIPEA, DMF, 85 °C, 14 h, 82–99%; (b) TFA, CH₂Cl₂, rt, 2 h, then Fe, AcOH, 100 °C, 4 h, 75–83% over 2 steps; (B) Synthesis of 4,5-dihydrobenzodiazepinone-based compounds 2, 3, and 82-84. (c) Pent-4-yn-1-yl methanesulfonylate, KI, DIPEA, DMF, μλ, 100 °C, 1 h, 62%; (d) HBPin, Cp₂ZrCl₂, DCE, 60 °C, 20 h, 72%; (e) 88/89, Pd(PPh₃)₂Cl₂, K₂CO₃, 1,4-dioxane, H₂O, 100 °C, 89–99%;
(f) Grubbs I catalyst, HSiEt₃, Tol, 40 °C, 2 h, 69%; (g) Vinilsilane, 1 M BCl₃ in heptane, CH₂Cl₂, 0 °C, 16 h, then 88, Pd(Amphos)₂Cl₂, K₂CO₃, toluene, THF, EtOH, H₂O, 100 °C, 80% over two steps; (h) 85, Pd(OAc)₂, Cu(I)I, PPh₃, NEt₃, 100 °C, 44%; (i) 71, DIPEA, DMF, 85 °C, 14 h, 66%; (j) TFA, CH₂Cl₂, rt, 2 h, then Fe, AcOH, 100 °C, 30 min, 44%; (k) H₂, 10% Pd/C, EtOH, rt, 3 h, 78%.

Replacement of the amide (65) for the (E)-alkene resulted in compound 2, which has a Kₐ = 102 ± 10 nM (ITC) for the CREBBP bromodomain (Table 4). Comparison of the ITC signature plot for 65 and 2 shows that the ΔH component is smaller for 65 than for 2, consistent with the hypothesis that the intramolecular hydrogen bond in 65 is being broken upon protein binding. Interestingly, the −TΔS term is smaller for 2 than for 65, suggesting that removal of the hydrogen bond does reduce some solution phase pre-organization of 2 (Figure S7A and B). As expected, the enantiomer, 3, showed no CREBBP bromodomain affinity. The (Z)-alkene- and alkyne-containing compounds (82 and 83) retain significant affinity for the CREBBP bromodomain with Kₐ = 0.329 ± 0.08 nM and Kₐ = 0.154 ± 0.03 nM (ITC), respectively. The increased affinity of the alkene, versus the amide 65, supports our hypothesis that the intramolecular hydrogen bond observed in 65 is detrimental to CREBBP bromodomain binding. It is also interesting that both the (Z)-alkene (82) and the alkyne (83) retain CREBBP bromodomain affinity. Molecular dynamics simulations (vide infra) predict that these compounds can be accommodated in the CREBBP KAc-binding site (Figure S9). Perhaps surprisingly, the alkane 84 shows no detectable affinity for the CREBBP bromodomain, indicating that some degree of structural pre-organization is required for CREBBP bromodomain affinity (Table 4).
Table 4. CREBBP and BRD4(1) $K_d$ values (ITC and NMR), $pK_d$ values, and CREBBP vs BRD4(1) selectivity for compounds 65, 2, 3, and 82-84. For ITC plots and binding isotherms see Figures S12 and S13.

| Compound number | Structure          | CREBBP $K_d$ (μM) (ITC) | CREBBP $pK_d$ | BRD4(1) $K_d$ (μM) (ITC) | BRD4(1) $K_d$ (μM) (NMR) | CREBBP / BRD4(1) selectivity |
|-----------------|--------------------|--------------------------|---------------|--------------------------|--------------------------|-----------------------------|
| 65              | ![Structure](image1) | 0.466 ± 0.03$^a$        | 6.3           | 2.82 ± 0.32$^a$          | 5.80 ± 0.99              | 6.1-12.4                    |
| 2               | ![Structure](image2) | 0.102 ± 0.01$^b$        | 7.0           | n.b.$^c$                 | 10.3 ± 1.89              | 101                         |
| 3               | ![Structure](image3) | n.b.$^d$                 | -             | n.t.$^e$                 | n.t.$^f$                 | -                           |
| 82              | ![Structure](image4) | 0.329 ± 0.08             | 6.5           | 1.17 ± 0.03$^g$          | 2.03 ± 0.41              | 4.3-6.2                     |
| 83              | ![Structure](image5) | 0.154 ± 0.03$^g$        | 6.8           | n.b.$^f$                 | n.t.$^f$                 | -                           |
| 84              | ![Structure](image6) | n.b.$^g$                 | -             | n.t.$^f$                 | n.t.$^f$                 | -                           |

n.t.: not tested; n.b.: no binding, ± standard error of the fit unless stated. A heatmap representation is used; high affinity is indicated by hot colors. $^a$n=2 ± s.d. from the mean. $^b$n=5 ± s.d. from the mean. $^c$n=4. $^d$n=2 ± s.d. from the mean. $^e$n=5 ± s.d. from the mean. $^f$n=2.

We initially tried to assess the CREBBP / BRD4(1) selectivity of 2 using ITC. However, under the conditions used, we were unable to detect binding to BRD4(1). Instead we used ligand-observed $^1$H NMR with protein titration (Figure S9), which gave estimated a $K_d$ values of 5.80 ± 0.99 μM for 65, 2.03 ± 0.41 μM for the (Z)-alkene 82, and 10.3 ± 1.89 μM for 2. Pleasingly, these data indicate that 2 is >100-fold selective for CREBBP versus BRD4(1).
Figure 10. A representation of the bromoKdELECT data for (A) compound 2 and (B) compound 3. Using the BROMOscan platform, $K_d$ values were determined for 12 phylogenetically-diverse bromodomains (ATAD2B, BRD2(1), BRD4(1), BRD7, BRD9, BRPF1, CREBBP, P300, PCAF, SMARCA4, TAF1(2), and TRIM24). Compound 2 showed $K_d$ values of 200 nM and 240 nM for the CREBBP and P300 bromodomains, respectively. No binding was seen at any of the other bromodomains at concentrations of up to 1 μM. Compound 3 showed no binding to any of the other bromodomains at concentrations of up to 1 μM.

To further assess the selectivity of 2 we subjected it to a BROMOscan to determine its $K_d$ values against a phylogenetically diverse panel of 12 bromodomains (Figure 10). In this assay, 2 showed $K_d$ values of 200 nM and 240 nM for the CREBBP and P300
bromodomains, respectively. No binding was seen at any of the other bromodomains at concentrations of up to 1 μM. In the same assay, compound 3 showed no binding to any of the bromodomains tested at concentrations of up to 1 μM. These data show that compound 2 is suitable for use in a cellular setting to probe the effect of inhibiting CREBBP and P300 bromodomain function.

ANALYSIS OF CREBBP BROMODOMAIN LIGAND BINDING AND SELECTIVITY
An X-ray crystal structure of 2 bound to the CREBBP bromodomain revealed that the 4,5-dihydrobenzodiazepinone headgroup bound as expected in the KAc-binding pocket. The full structure is, however, more complex, with 7 proteins observed in the unit cell (Figure 11A). Surprisingly, the THQ group formed an interaction with the R1173 residue of an adjacent protein, resulting in a dimeric structure with two ligands bound to two bromodomains (Figure 11B). We used size-exclusion chromatography (SEC) to determine whether the dimeric structure occurred in solution or is a crystallographic artefact. This method has been used by others for the verification of bivalent ligands. These experiments indicated that dimerization is not occurring in solution as an identical peak was observed in both the presence or absence of compound 2 (50 μM, Figure S10).
**Figure 11.** An X-ray crystal structure of compound 2 bound to the CREBBP bromodomain (PDB code 6YIJ). **A.** Seven chains are observed in the unit cell. **B.** The bromodomains form dimeric units in the crystal structure, with the ligand that is bound to chain A interacting with the R1173 residue of the cognate chain B. See Figure S11B for ligand electron density map.

Given the SEC results, we used molecular dynamics simulation to predict the monomeric structure of 2 bound to a single CREBBP bromodomain. Starting from a single bromodomain, taken from the X-ray crystal structure (PDB code 6YIJ), molecular dynamics simulations (50 ns run in triplicate, Figure 12) predict that the THQ group moves closer to P1110 settling at a distance of ≈4.2 Å. This residue is located below R1173, supporting the hypothesis that 2 has a similar binding mode to compounds 1 and 65 when bound to the CREBBP bromodomain.
Figure 12. **A.** MD simulations predict that the electrostatic interaction between 2 and R1173 is present in solution. **B.** The distance between R1173 and the π-system of 2 drops to below 7 Å during equilibrium MD simulations meaning that the pose observed in the X-ray crystal structure (PDB code 6YIJ) relaxes to give a conformation that is similar to that observed for 65 bound to the CREBBP bromodomain (PDB code 6YIM, Figure 7). Simulations were carried out in triplicate.

Docking studies on the (Z)-alkene 82 and the alkyne 83 (Figure S8) predict that these compounds bind to the CREBBP bromodomain in a similar conformation to 65. As with compound 2 (Figure 12A), molecular dynamics simulation (50 ns run in triplicate) were carried out starting from either the X-ray protein crystal structure for compound 65 (PDB code 6YIM), or the docked structures for compounds 82 and 83. In all cases these simulations predict that the THQ group resides close to P1110 and in the proximity of R1173. The fact that compounds 82 and 83 can form the same interaction as 2 is consistent with their measured binding affinity for the CREBBP bromodomain.
RATIONALISING THE CREBBP vs BRD4(1) SELECTIVITY

We were particularly intrigued as to how the modest change from an amide to an alkene resulted in such a substantial increase in selectivity for CREBBP versus BRD4(1). An X-ray crystal structure of the amide 65 bound to BRD4(1) was key to our understanding of this observation. This structure revealed that 65 adopts a very different conformation when bound to BRD4(1) compared to when it is bound to the CREBBP bromodomain (Figure 13). When bound to CREBBP the ligand adopts a curved conformation, so as to interact with P1110 and R1173; when bound to BRD4(1) it adopts an extended conformation resulting in London dispersion forces between the THQ ring, W81 and L92. This type of conformation has been observed with other BET bromodomain ligands, including I-BET762 and BI-2536.56

Figure 13. An overlay of the X-ray crystal structure of compound 65 bound to BRD4(1) (PDB ID: 6YIN. carbon = pink) and the X-ray crystal structure of compound 65 bound to the CREBBP bromodomain (PDB ID: 6YIM. carbon = purple). The surface of BRD4(1) is shown in white. The conformation of 65 when bound to BRD4(1) is more extended than
that when bound to the CREBBP bromodomain. This conformation allows 65 to interact with W81, which likely contributes to its affinity for BRD4(1).

We reasoned that the low affinity of 2 for BRD4(1) indicates that it cannot adopt the same conformation as 65 when bound to this bromodomain. Docking studies indicated that 2 can initially bind to BRD4(1) in a similar orientation to 65, but 25 ns MD simulations show that the linker of 2 did not hold a stable orientation relative to the 4,5-dihydrobenzodiazepinone headgroup. The key to this observation lies in the interactions with the BRD4(1) ZA channel water molecules. A 25 ns MD simulation starting from X-ray crystal structure of 65 bound to BRD4(1) (Figure 15A) predicts a high density of water in the ZA channel. This observation is consistent with the presence of a hydrogen bond between the amide carbonyl oxygen atom of 65 and the BRD4(1) ZA-channel water molecule, which is observed in the X-ray crystal structure (PDB code 6YIN). In the simulation of 2 bound to BRD4(1) there is no water density present in the same region. This indicates that this water molecule would have to be displaced for 2 to bind to BRD4(1), presumably because 2 cannot form a hydrogen bond. As this water molecule is very hard to displace, compound 2 is unable to bind to BRD4(1), which is reflected in its very low measured affinity.

Interestingly, docking of the (Z)-alkene 82, which shows low μM affinity for BRD4(1), identified two poses where the THQ could reside against either L92 or W81. Both poses were subject to MD studies, which showed both locations of the THQ group allowed it to form interactions with L92, W81 and P82. Both of these poses hold the (Z)-alkene bond perpendicular to the KAc mimic and therefore do not result in displacement of both ZA
channel water molecules (Figure 14C). It seems, therefore, that ligands that can interact with or accommodate this water molecule will retain affinity for BRD4(1), whereas those that cannot, including 2, are not able to bind to BRD4(1), resulting in high selectivity over this bromodomain.

**Figure 14.** Snapshots of molecular dynamics simulations (taken at 25 ns) starting from the (A) X-ray crystal structure of 65 (carbon = purple) bound to BRD4(1) (PDB code 6YIN), (B) the structure of 2 (carbon = blue) docked to BRD4(1) following a 25 ns MD simulation, or (C) the structure of 82 (carbon = teal) docked to BRD4(1) following a
25 ns MD simulation. Crystallographic water molecules from BRD4(1) (PDB code 6YIN) are shown as red spheres. Areas of high water density are shown as a blue mesh. In A and C the water densities overlay the crystallographic water molecules, indicating that this binding pose is stable. In B, no water density is observed, meaning that the crystallographic water molecules would have to be ejected to accommodate binding of 2. As displacement of the crystallographic water molecules is likely to be unfavorable, 2 does not bind to BRD4(1), resulting in the high selectivity of this compound for the CREBBP bromodomain over BRD4(1).

THE EFFECTS OF CREBBP AND P300 BROMODOMAIN INHIBITION IN HCT116 CELLS
With a $K_d$ value of $102 \pm 10$ nM, high selectivity over BRD4(1), and no effects on a phylogenetically-diverse panel of bromodomains, compound 2 is suitable for use as a probe of CREBBP bromodomain function in cells.
Figure 15. Inhibition of CREBBP/P300 with 2 reduces c-Myc production and acetylation of H3K18 in HCT116 cells (human colorectal cancer cell line), and stabilizes HIF-1α in hypoxia. A. HCT116 cells were exposed to 2 (10 μM) for the times shown. Western blots were then carried out for the proteins indicated. β-Actin is included as a loading control. B. HCT116 cells were exposed to 2 (10 μM) for the times indicated followed by mRNA preparation. Q-PCR was then carried out to determine c-Myc mRNA levels compared to the 18S. Data were analyzed using one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. C. The inactive compound 3 does not reduce levels of c-Myc. D. HCT116
cells were exposed to compound 2 (10 μM) in hypoxia for the times shown. c-Myc was reduced in hypoxia, which is a known phenomenon. Stabilization of HIF-1α above the level observed in hypoxia alone was observed.

Treatment of human colorectal cancer cells (HCT116) with 2 (10 μM) reduces expression of c-Myc after 6 h, with more substantial effects observed after 16 h and 24 h (Figure 15A). These effects were also observed on c-Myc mRNA (Figure 15B), confirming that inhibition of the CREBBP/P300 bromodomain affects c-Myc at the transcriptional level. Treatment of the same cells with the inactive enantiomer, 3 (10 μM), had no effect on c-Myc expression after 24 h (Figure 15C), supporting the hypothesis that these effects occur as a result of CREBBP/P300 bromodomain inhibition. These data are consistent with previous reports that selective inhibition of the CREBBP bromodomain results in reduced levels of c-Myc.15-18,57

In addition to modulating c-Myc levels, we also observed that treatment of HCT116 cells with 2 (10 μM) caused a modest, but reproducible, decrease in acetylation of H3K18 (Figure 15A). In contrast, Zucconi et al.,58 observed an increase in H3K18Ac in an acute myeloid leukemia line (KG1a) and an androgen-dependent prostate cancer cell line (LNCaP), on treatment with the CREBBP/P300 bromodomain ligand I-CBP112 (10 or 20 μM). Interestingly this effect was not observed on treatment with CPB30. The reason for the differences in these data is not clear, and it should be noted that different cell lines are used, but this hints at some CREBBP/P300 bromodomain ligands having an allosteric modulatory effect on the KAT activity of CREBBP/P300. This is consistent with our PrOF
NMR studies (vide supra), which showed shifts in the fluorine signals in opposite directions for 2 and I-CPB112, suggesting that different allosteric effects could be possible depending on the structure of the bromodomain ligand. It is possible that CPB30, which possesses the smaller 3,5-dimethylisoxazole KAc mimic,\textsuperscript{13,59-61} does not cause an allosteric effect on the KAT domain.

In hypoxia (<0.1% O\textsubscript{2}), we were intrigued to observe that treatment of HCT116 cells with 2 (10 μM) led to stabilization of HIF-1α above the level observed in hypoxia alone (Figure 17D). While it has long been known that CREBBP/P300 bind to HIF-1α,\textsuperscript{8} the mechanism by which this stabilization occurs is currently unclear. It has previously been shown that inhibition of the BET bromodomains with (+)-JQ1 does not affect HIF expression levels or activity,\textsuperscript{62} indicating that 2 selectively inhibits the CREBBP/P300 bromodomains in cells, resulting in this effect. This observation potentially offers a novel route for the intervention in clinical conditions affected by HIF-1α levels. Particularly relevant examples of such conditions are the SARS-CoV and SARS-CoV-2 (COVID-19) viruses.\textsuperscript{63} Both viruses use angiotensin-converting enzyme 2 (ACE2), a zinc metalloprotease, as the spike protein receptor to enable virus entry into host cells. ACE2, along with its homolog ACE, is a component of the renin-angiotensin system, which processes angiotensin peptides to regulate cardiovascular function. In hypoxia, expression levels of ACE2 have been reported to be reduced in the long term.\textsuperscript{68} This results from a HIF-1α-mediated increase in ACE expression, leading to an increase in angiotensin II (Ang II, angiotensin 1-8). Ang II, in turn, activates Ang II type 1 receptors, which decrease ACE2 production.\textsuperscript{68} It has been suggested that increased levels of ACE2 might correlate with increased SARS-CoV-2 infection.\textsuperscript{69} Use of protease inhibitors, to prevent SARS-CoV-2 spike protein
binding to ACE2, has proved effective at reducing SARS-CoV-2 infection of human cells.\(^6\) A complementary strategy could be to transiently reduce ACE2 expression through modulation of HIF-1α levels, while at risk from SARS-CoV-2 infection. While further work is clearly needed to test this hypothesis, inhibition of the CREBBP bromodomain might provide a route to transiently stabilize HIF-1α, reduce levels of ACE2 expression, and therefore lower susceptibility to SARS-CoV-2 infection.

In conclusion, we report the extensive SAR studies that led to the development of a high affinity ligand for the bromodomains of CREBBP and P300. A key aspect was the understanding that the intramolecular hydrogen bond present in \(65\) predisposed the molecule to adopt a solution phase conformation that is unfavorable for binding to the CREBBP/P300 bromodomains. Replacement of the amide with a \((E)\)-alkene led to the high affinity ligand \(2\) \((-\)-OXFBD05). This ligand does not show appreciable affinity at any of the bromodomains we evaluated, including BRD2(1) and BRD4(1), making it suitable for use in cellular studies. Initial work shows that inhibition of the CREBBP/P300 bromodomain results in downregulation of c-Myc in HCT116 colon cancer cells. A reduction in H3K18 acetylation is also observed, demonstrating that the bromodomain plays a role in the KAT function of CREBBP/P300. In addition, stabilization of HIF-1α above the level observed in hypoxia alone was seen; the mechanism of this effect is currently unknown. \((-\)-OXFBD05 \((2)\), and its inactive enantiomer companion compound \((+\)-OXFBD05 \((3)\), will likely be useful tools for those interested in studying the CREBBP/P300 bromodomain. Beyond its specific focus, our study provides wider insight into the use of intramolecular hydrogen bonds to manipulate the solution state conformation of molecules. We have paid particular attention to comparing the presence
of intramolecular hydrogen bonds in solution and when bound to the CREBBP bromodomain. This general approach potentially offers the ability to design compounds that have favorable entropic properties for protein binding, while retaining useful physicochemical properties. Consequently, this work will be of broad interest to those engaged in the design of probe and drug molecules.
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