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Coordination complex SH2 domain proteomimetics: an alternative approach to disrupting oncogenic protein–protein interactions†

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Abstract

We report the first application of coordination complexes as functional proteomimetics of the Src homology 2 (SH2) phosphopeptide-binding domain. As a proof-of-concept, functionalized bis-dipicolylamine (BDPA) copper(II) complexes are shown to disrupt oncogenic Stat3–Stat3 protein complexes and elicit promising anti-tumour activity.

Inhibition of cancer-promoting, constitutive protein–protein complexes *via* disruption of binding interfaces offers significant value as a molecular-targeted therapy.¹ Protein–protein interactions are frequently initiated and maintained through phosphorylation of critical tyrosine (Y) or serine amino acid residues. Proteins containing key phosphorylated Y residues are often recognized and bound by specific phosphopeptide-binding modules, *e.g.* the Src homology 2 (SH2) domain, of the complementary protein-binding partner.² Despite there being two valid targets for the intervention of such interactions—the phosphopeptide and the phosphopeptide-binding module—the majority of effort has focused on the development of phosphopeptide mimetics.^{3–5} However, these agents typically incorporate phosphate groups, leading to pharmacokinetic drawbacks associated with their polarity and lability.⁶ It is surprising that, with the exception of one important example,⁷ there have been no reports of tackling the problem from the alternative direction; that is, through mimicry of the phosphopeptide recognition module.

We sought to determine whether oncogenic protein–protein complexes mediated by SH2 domain–phosphotyrosine (pY) interactions could be disrupted by replicating the function of the SH2 domain in small molecules. SH2 domain–pY interactions play a key role in the

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oncogenic transcriptional activity of signal transducer and activator of transcription 3 (Stat3) protein.¹ Stat3 is a master regulator of the underlying events in malignant transformation.^{8,9} In order to become transcriptionally active, Stat3 protein must homodimerize. Hence, numerous groups, including ours,¹⁰ have attempted to silence aberrant Stat3 activity through disruption of the Stat3–Stat3 complex.^{10,11} To date, Stat3 inhibitors have focused primarily upon targeting the Stat3-SH2 domain ‘hot spot’, mimicking key elements of the SH2 domain recognition sequence. However, the large, planar interfacial areas involved in protein-binding interfaces, such as the SH2 domain, render them daunting targets.¹² Traditional drug discovery approaches targeting the SH2 domain have failed, thus far, to discover a truly potent Stat3 inhibitor.¹³ Recently, Hamachi *et al.* demonstrated *in vitro* that phosphate binders such as Lewis acidic metal–picolylamine complexes could disrupt a phosphopeptide recognition module (Pin1 WW)–phosphopeptide complex.⁷ We reasoned that this approach could be adopted to develop SH2 domain proteomimetics.

We herein report the first application of coordination complexes as functional proteomimetics of SH2 domains. In a novel therapeutic role, bis-dipicolylamine (BDPA) copper(II) complexes are shown to inhibit phosphopeptide–Stat3 complexes, disrupt Stat3–Stat3 protein complexes in cancer cell extracts and elicit promising anti-tumour activity.

Since the most critical recognition component of an SH2 domain is the phosphate-binding moiety, all of our mimetics incorporate Lewis acidic metals as a key structural feature.¹⁴ Considerable research has been conducted on the BDPA ligand which was, therefore, an obvious choice for this work.¹⁵ In addition, we were keen to investigate the scaffold linking the two DPA sub-structures and proposed proteomimetics possessing the flexible 1,5-propyl (**1**; Fig. 1)¹⁶ and the more rigid *m*-xylyl (**2** and **3**)¹⁷ linkages. Beyond pY recognition, these structures offer little potential for interactions with the residues flanking the pY. However, deriving phosphopeptide specificity was not a key objective of this work, rather, our goal was to establish whether a pY binder could disrupt an oncogenic protein–protein interaction. Given significant precedence for sequestering phosphate anions when complexed to DPA ligands, Cu(II) was selected as the Lewis acidic guest.¹⁴

We first considered the binding potency of the individual agents for the Stat3 phosphorylated binding sequences. Through its SH2 domain, Stat3 is recruited to specific pY residues located on Stat3 and glycoprotein 130 (gp130), as well as many other critical mediators. In this study, the binding potency of inhibitors **1–3** was assessed against these two binding partners. Titration experiments were performed on a MicroCal VP-ITC at physiological pH 7.4 (5 mM substrate, 0.25 mM of ligand); a typical trace is given in Fig. 2. Inhibitors **1–3** displayed reasonable binding affinity for the respective native Stat3 and gp130 phosphorylated sequences Ac-pYLKTK–NH₂ and Ac-pYLPQTV–NH₂ (Table 1), with *K*_d values in the range of 8–100 μM (Stat3 SH2 domain–Ac-pYLKTK–NH₂, IC₅₀ ≈ 39 μM).¹⁸

It is important to note that complexes have been used to hydrolyze phosphate esters,¹⁹ however, the characteristic binding isotherms obtained showed that ester hydrolysis of the pY residue was not a factor in the experimental time frame (1 h).²⁰ Given the affinities obtained for the two phosphopeptides (Table 1), we were confident that our mimetics would be sufficiently tight binders to compete with the Stat3-SH2 domain and disrupt the oncogenic Stat3–Stat3 complex.

Disruption of the Stat3 protein–phosphopeptide interaction with mimetics **1–3** were assessed by a competitive fluorescence polarization (FP) assay popularly used to determine the Stat3-SH2 domain affinity of small molecules. The assay is based upon the displacement of a 5-carboxyfluorescein-labeled (F*) gp130 phosphopeptide, *i.e.* F*pYLPQTV, from the Stat3-

SH2 domain, wherein unbound F*pYLPQTV results in reduced polarization of the emitted fluorescence due to a higher mobility of the fluorophore. We reasoned that a Stat3-SH2 domain mimetic would compete with Stat3 (90 kDa) for the phosphopeptide and result in a significantly lower molecular weight binding complex (mimetic-F*pYLPQTV *cf.* Stat3-F*pYLPQTV)—dramatically lowering the observed polarization. The FP assay was performed similarly to that described by Berg *et al.*^{18,21} The formation of the Stat3-F*pYLPQTV complex was verified on this set up ($K_d = 142 \pm 42$ nM).¹⁸ For the inhibition experiments, the Stat3-F*pYLPQTV complex gave a starting FP value of 120–140 mP. Treating the Stat3-F*pYLPQTV complex with inhibitors resulted in concentration-dependent decreases in polarization, related to the dissociation of the phosphopeptide from the Stat3 protein. Typical titration data are shown in Fig. 3. The K_i values are listed in Table 2.

Compounds **2** and **3** showed encouraging disruption of the protein–phosphopeptide complex, with K_i values of 15 μ M and 38 μ M, respectively, whilst mimetic **1** demonstrated slightly lower inhibitory activity ($K_i = 128$ μ M). Comparing the data for **1** with **2** and **3** suggests that functionalization of the DPA periphery as in **2** and **3** appears to enhance activity. Whilst **2** exhibited the most potent inhibitory action ($K_i = 15$ μ M), the FP data suggest that the mode of disruption is more complex than that of the other inhibitors. The high concentration limit of FP is *ca.* 73 mP for **2**, compared to 30 mP for all other compounds. We presume that higher order phosphopeptide–inhibitor complexes are formed. Anslyn has previously reported aggregate binding phenomena.²² Excepting **2**, compounds **1** and **3** exhibited greater decreases in anisotropy, with typical high concentration FP limit values (~ 30 mP) approximately twice as large as the value of the free peptide (15 mP). Since the FP depends approximately linearly on the mass of the molecule,²³ this suggests the formation of a peptide–inhibitor complex (ESI[†]).

Highly encouraged by the ITC and FP data, we sought to determine whether our proteomimetics could disrupt a transcriptionally active protein complex such as Stat3–Stat3. Thus as described in the literature, we treated the nuclear extracts from cells containing constitutively activated Stat3 (NIH3T3/v-Src (v-Src-transformed mouse fibroblasts))²⁴ with inhibitors **1–3** for 30 min, and subsequently measuring the Stat3–Stat3 : DNA binding *in vitro* using an excess of Stat specific radiolabeled hSIE probe and electrophoretic mobility shift assay (EMSA) we showed significant levels of proteomimetic-induced Stat3 dimer disruption (Fig. 4A).²⁵ Excitingly, inhibitor **1** suppressed Stat3–Stat3 : DNA binding with an IC_{50} value of 61 ± 4.4 μ M, inhibiting Stat3 complexes and thus preventing DNA binding. Inhibitors **2** and **3** displayed similar levels of Stat3 disruption with IC_{50} values of 74 μ M and 41 μ M, respectively.

In order to confirm that our mimetics were not interacting with the DNA probe, several control experiments were conducted. The EMSA assay was repeated using nuclear extracts containing both activated Stat1 and Stat3 proteins prepared from EGF-stimulated NIH3T3/hEGFR fibroblasts (Fig. 4B). Treating NIH3T3/hEGFR nuclear extracts containing Stat3–Stat3, Stat1–Stat3 and Stat1–Stat1 protein complexes with **1** provided an opportunity to visualize isoform-selective Stat disruption. Inherently this assay would also determine whether the inhibitor was directly disrupting the protein complexes or interfering with the DNA probe thus preventing ternary complex formation. If inhibitors interacted exclusively with the DNA, we would expect equal inhibition of all three protein–DNA complexes. Although at this stage it is not possible for us to comment on the specificity of our inhibitors for the Stat proteins relative to other phosphorylated proteins, we found that inhibitor **1**

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displayed isoform selectivity for Stat3 over Stat1 protein complexes; mimetic **1** showed a three-fold preference for Stat3 (Fig. 4B: **1**, Stat3 IC_{50} = 61 ± 4 cf. Stat1 IC_{50} = 176 ± 24 μ M). We also considered that the BDPA ligand might itself be acting as an intercalating agent. However, EMSA analysis with unchelated ligands conferred negligible effects upon Stat3–Stat3 : DNA binding (NIH3T3/vSrc, IC_{50} > 300 μ M). These results suggest that a protein specific inhibitory mode of action is responsible for the disruption of the ternary complex and not simply inhibitor-mediated blockage of the DNA probe. Taken in conjunction with the ITC and FP data, our EMSA data suggest that mimicry of the SH2 domain function may be the principle mode of inhibition.

To determine the therapeutic potential of our mimetics, we screened against a range of cancer cell lines known to contain activated Stat3 protein, including prostate cancer (DU145), acute myeloid leukemia (OCI-AML) and breast cancer (MDA468) (Table 3). Screening identified **2** as a promising anti-cancer agent, exhibiting low μ M activity against all three cell lines. Viability studies with $Cu(II)(OTf)_2$ and free ligand showed negligible cytotoxic effects. Whilst suppression of Stat3 containing tumour cell viability does not prove whole cell Stat3 specificity, *in vitro* whole cell EMSA analysis of the nuclear extracts of NIH3T3/vSrc cells treated with inhibitors showed suppression of Stat3 complexes. Encouragingly, lower cytotoxicity was observed in healthy NIH3T3 cells treated with inhibitors (all whole cell data shown in ESI[†]).

In conclusion, we have presented evidence to suggest that coordination complexes can act as disruptors of clinically relevant protein–protein interactions. We postulate that the inhibitory action is based upon mimicry of the SH2 domain's pY binding function. Our group is now focused on developing specific phosphopeptide recognition scaffolds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

1. Burke WM, Jin X, Lin H-J, Huang M, Liu R, Reynolds KR, Lin J. *Oncogene* 2001;20:7925–7934. [PubMed: 11753675]
2. Waksman G, Kominos D, Robertson SC, Pant N, Baltimore D, Birge RB, Cowburn D, Hanafusa H, Mayer BJ, Overduin M. *Nature* 1992;358:625–626. [PubMed: 1379695]
3. Muller G. *Top. Curr. Chem* 2000;211:17–59.
4. Gunning PT, Glenn MP, Siddiquee KAZ, Katt WP, Masson E, Sebt SM, Turkson J, Hamilton AD. *ChemBioChem* 2008;9:2800–2803. [PubMed: 19006150]
5. Siddiquee KAZ, Gunning PT, Glenn MP, Katt WP, Zhang S, Schroeck C, Sebt SM, Jove R, Hamilton AD, Turkson J. *ACS Chem. Biol* 2007;12:787–798. [PubMed: 18154266]
6. Burke TRJ, Russ P, Lim B. *Synthesis* 1991:1019–1020.
7. Ojida A, Inoue M, Mito-oka Y, Tsutsumi H, Sada K, Hamachi I. *J. Am. Chem. Soc* 2006;128:2052–2058. [PubMed: 16464107]
8. Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, Nam S, Eweis I, Diaz N, Sullivan D. *Clin. Cancer Res* 2006;12:20–28. [PubMed: 16397019]
9. Zamo A, Chiarle R, Piva R, Howes J, Fan Y, Chilosi M, Levy DE, Inghirami G. *Oncogene* 2002;21:1038–1047. [PubMed: 11850821]

10. (a) Fletcher S, Turkson J, Gunning PT. *ChemMedChem* 2008;3:1159–1168. [PubMed: 18683176]
(b) Fletcher S, Jardephi J, Yue P, Page BDG, Shahani VM, Sharmeen S, Schimmer AD, Turkson J, Gunning PT. *ChemBioChem* 2009;10:1959–1964. [PubMed: 19644994]
11. Berg T. *ChemBioChem* 2008;9:2039–2044. [PubMed: 18677740]
10. (a) Fletcher S, Hamilton AD. *Curr. Top. Med. Chem* 2007;7:922–927. [PubMed: 17508923] (b) Fletcher S, Hamilton AD. *J. R. Soc. Interface* 2006;3:215–233. [PubMed: 16849232]
13. Yue P, Turkson J. *Expert Opin. Invest. Drugs* 2009;18:45–56.
14. Kruppa M, Konig B. *Chem. Rev* 2006;106:3520–3560. [PubMed: 16967915]
15. Ojida A, Mito-oka Y, Sada K, Hamachi I. *J. Am. Chem. Soc* 2004;126:2454–2463. [PubMed: 14982454]
16. Yashiro M, Ishikubo A, Komiyama M. *J. Biochem* 1996;120:1067–1069. [PubMed: 9010750]
17. Drewry JA, Fletcher S, Hassan H, Gunning PT. *Org. Biomol. Chem* 2009;7:5074–5077. [PubMed: 20024100]
18. Schust J, Berg T. *Anal. Biochem* 2004;114–118. [PubMed: 15183768]
19. Chin J. *Curr. Opin. Chem. Biol* 1997;1:514–521. [PubMed: 9667897]
20. (a) Benniston AC, Gunning PT, Peacock RD. *J. Org. Chem* 2005;70:115–123. [PubMed: 15624913] (b) Gunning PT, Benniston AC, Peacock RD. *Chem. Commun* 2004:2226–2227.
21. Liu B, Fletcher S, Avadisian M, Gunning PT, Gradinaru C. *J. Fluoresc* 2009;19:915–920. [PubMed: 19459035]
22. Rekharsky M, Inoue Y, Tobey S, Metzger A, Anslyn E. *J. Am. Chem. Soc* 2002;124:14959–14967. [PubMed: 12475338]
23. Lakowicz, JR. *Principles of Fluorescence Spectroscopy*. 3rd edn. New York: Springer; 2006.
24. Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, Jove R. *Mol. Cell. Biol* 1998;18:2545–2552. [PubMed: 9566874]
25. Turkson J, Ryan D, Kim JS, Hamilton AD, Sehti SM, Jove R. *J. Biol. Chem* 2001;276:45443–45455. [PubMed: 11579100]

Fig. 1.
Chemical structures of SH2 domain proteomimetics **1**–**3**.

Fig. 2.
A representative ITC for **1** binding to the Stat3 sequence pYLKTK ($30 \times 10 \mu\text{L}$ injections), 50 mM HEPES, pH 7.4, 25 °C.

Fig. 3. FP measured upon titrating the F*pYLPQTV (7.2 nM) with compounds **1** and **3** in the presence of Stat3 protein (292 nM).

Fig. 4.
A. EMSA of Stat3 dimerization inhibition (as judged by the disruption of the Stat3–Stat3 : DNA complex) *via* **1** and **2**. Positions of Stat3–Stat3 : DNA complexes in gel are shown; data are representative of 3 independent assays; B. EMSA analyses of Stat isoform selectivity of **1**.

Table 1

Binding constant (K_d , μM) for interactions between SH2 mimetics and phosphorylated Stat3 binding sequences

Peptide	1	2	3
pYLKTK (Stat3)	8.2 ± 1	23 ± 2.2	43 ± 5.6
pYLPQTV (gp130)	— ^a	100 ± 31	43 ± 3.7

^aUnable to fit binding isotherm to a 1 : 1 binding model.

Table 2

K_i values for inhibitors **1–3** obtained from the fit of FP data

Inhibitor	1	2	3
$K_i/\mu\text{M}$	128 ± 15	15 ± 9	38 ± 7

Table 3

IC₅₀ values (mM) for inhibitors in whole cell viability studies in DU145 (prostate cancer) OCI-AML-2 (acute myeloid leukemia) and MDA468 cell lines (breast cancer)

Inhibitor	1	2	3
DU145	76.6 ± 15	11.5 ± 4.2	>100
OCI-AML2	73.1 ± 25	11.3 ± 6.3	>100
MDA468	11.5 ± 4	5.0 ± 0.5	56