1 **Identification of a Potent Activator of Akt Phosphorylation from**
2 **a Novel Series of Phenolic, Picolinic, Pyridino and Hydroxamic**
3 **Zinc(II) Complexes**

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Abstract

Discovery of small molecule modulators of signalling pathways is currently a particularly active area of research. We aimed at developing unprecedented metal-based activators of Akt signalling, which can potentially find applications as tools for regulating glucose metabolism downstream of Akt or serve as lead structures for developing anti-diabetic drugs. In this context, a highly diverse library of eleven new zinc(II) complexes with phenolic, picolinic, pyridino and hydroxamic ligands, all containing features beneficial for medicinal purposes, was prepared and screened in an assay that detected levels of phospho-Akt in lysates from NIH3T3 cells after treatment with the compounds. The complexes featuring hydroxamic-based ligands were found to be the most prominent activators of Akt among the prepared molecules, with the most efficient compound (**11**) acting at sub-micromolar concentrations. Further characterisation revealed that this compound induces phosphorylation of the Akt downstream effector glycogen synthase kinase 3 β (GSK3 β), but does not act as an inhibitor of tyrosine phosphatases or PTEN.

Introduction

Small molecules with the ability to modulate cellular signalling have the potential to act as spatial and temporal perturbagens of biological processes, and thus constitute a reversible, practical, easily tuned and readily accessible alternative to genetic mutations in our study of biological systems.^[1] Using chemical tools to interfere with processes that involve protein phosphorylation, for example, has a high likelihood of leading to significant biological consequences (both structural and functional),^[2] since it is now well established that in all living systems phosphorylation serves as a universal signal that marks the activation or deactivation of enzymes and signalling cascades.^[3]

A particularly appealing target for modulation is the serine/threonine kinase Akt. The activation of Akt (Figure 1) is due to the phosphorylation of two sites (Thr-308 and Ser-473) by protein kinases that are controlled by phosphoinositide-3-kinase (PI3K).^[4] PI3K is activated by upstream signals such as insulin binding to its receptor. Numerous reports have linked Akt phosphorylation to critical biological events such as cell growth, proliferation and survival,^[5] thus rendering it medicinally significant for cancer^[6] and neurological diseases^[7] among others. Akt activation has also been implicated in cellular glucose uptake and utilization, consequently Akt is also a highly relevant target in the development of anti-diabetic drugs.^[8]

Identification of new small molecules that make it possible to regulate at will the phosphorylation levels of Akt might yield control over some of these downstream processes. With a focus on the diabetes implications of Akt activation, we have previously shown that a series of vanadium(IV) and vanadium(V) complexes with picolinic ligands were able to induce Akt phosphorylation, acting by inhibiting PTEN phosphatase, an enzyme upstream of

Akt that directly counteracts the function of PI3K.^[8a] In parallel, other laboratories have suggested that protein tyrosine phosphatases (PTP) are possible targets for complexes of vanadium.^[9] Recent studies by Sakurai describing metal-based small molecules with the ability to enhance Akt phosphorylation levels have involved zinc(II) as the metal center.^[10] Most of these reports have suggested that zinc(II) complexes carry out their effects in a PI3K-dependent mode,^[11] and that relatively high concentrations of complexes are needed in order to achieve significant Akt phosphorylation enhancements.

Figure 1. Simplified schematic representation of the biochemical pathways that connect the insulin receptor (IR), PI3K and PTEN with Akt and its downstream effector GSK3β.

Encouraged by the above findings and given the relatively limited number of zinc(II) compound classes evaluated as Akt activators so far, we investigated a broader range of zinc(II) compound families, with the aim of identifying novel and more potent small molecules that can find applications as chemical perturbagens of Akt signalling and glucose metabolism. Zinc(II) favorably exhibits low toxicity (especially in comparison to vanadium) as well as the ability to afford complexes with a variety of coordination geometries, spanning

from tetra- to hexa-coordination. These properties were taken into account in our compounds design, along with the inclusion of new phenolic, picolinic, pyridino and hydroxamic ligands, that we considered likely to contribute favorable properties in terms of functional and structural diversity, H-bonding ability, aqueous solubility, membrane transport and pharmacokinetics. The synthesis of eleven new zinc(II) complexes (Figure 2) and their biological evaluation leading to identification of a potent Akt activator is described herein.

Figure. 2 Proposed structures of all zinc(II) coordination compounds prepared in this study.

90 **Results and Discussion**

91 **Synthesis of Ligands and Complexes**

92 As part of our effort to discover new and effective metal-based activators of Akt signalling,
93 we prepared an extended series of zinc(II) complexes, which would be inclusive of a diverse
94 set of ligands. The compound design had to fit certain requirements: the complexes should
95 exhibit distinct coordination geometries and 3D-shapes; exhibit various patterns of hydrogen
96 bond donors and acceptors; be inclusive of pharmaceutically-beneficial functionalities; have
97 good aqueous solubility and membrane permeability; and be readily synthesized. We chose to
98 investigate four major classes of ligands, namely phenolic, picolinic (similar to the ones we
99 previously evaluated with vanadium), pyridino and hydroxamic. The functional groups
100 present in these ligands were deemed compatible with all of the above criteria, based on their
101 performance in a multitude of previous pharmaceutical applications. Eleven complexes were
102 synthesized in total, all of which are novel compounds.

Scheme 1. Synthesis of phenolic zinc(II) complexes **1-4** (yields: **1**, 99%; **2**, 54%; **3**, X%; **4**, 95%)

The first group of complexes (compounds **1-4**, Scheme 1) includes polydentate Schiff base ligands (or their reduced counterparts) derived from salicyl precursors, and were formed using $\text{Zn}(\text{OAc})_2$ as the metal source. Compounds **1** and **2** exhibit an O-N-O coordination pattern in their tridentate ligand. Compound **1** was obtained by condensation of glycine on the 2-position of 2,4-di(formyl)-phenol in the presence of the metal, followed by a second condensation of the remaining aldehyde moiety to an ethanolamine unit. Complex **2** was obtained in one step by reacting an α -aminoacid, homocysteic acid, with a similar salicyl aldehyde that features a sulfonic acid substituent on the 4-position. The chosen polar functionalities in these compounds confer desired hydrophilicity and H-bonding ability to the complexes, both beneficial for their aqueous solubility and potential to interact with biomolecules. As suggested by our study and in agreement with a reported crystal structure of **1a**,^[12] complexes **1** and **2** were isolated in a hydrated state and in aqueous solution can

potentially reach hexa-coordination through further hydration. The fact that zinc(II) has no specific preference for a fixed geometry allows water to occupy free coordination sites, which is likely the case for other complexes in this study as well. Ligands for the synthesis of **3** and **4** were prepared from the same aromatic precursor as **2**. In the case of **3** (which has a ligand-to-metal stoichiometry of 2:1) the organic precursor was modified via reductive amination with n-propylamine to afford the N-O bidentate amine ligand **3a** prior to complexation. The ligand in **4** (which exhibits an O-N-N-O coordination pattern) links two precursor molecules via double imine formation upon their condensation with ethylenediamine. The complex likely has distorted pyramidal geometry, as has been demonstrated previously for other zinc(II)-salen complexes.^[13]

Scheme 2. Synthesis of picolinic zinc(II) complex **5** (yield: 34%)

Another bidentate N-O ligand was derived from 5-aminopicolinic acid (Scheme 2). Although this specific complex has not been previously reported, it should be noted that zinc(II) complexes with other derivatives of picolinic acid have been studied previously as insulin mimetics.^[14] The amine handle was initially modified to afford benzylamine **5a**^[15] in order to explore the impact of this bulky moiety on the biological activity of the corresponding zinc(II) complex. Complex **5** was prepared by reacting the ligand with zinc sulfate under basic conditions.

137 A group of four ligands were derived from 2-formylpyridines (Scheme 3) via imine
 138 formation. In the case of compounds **6b** and **7b** (ligands for **6** and **7** respectively) the amine
 139 components reacting with 2-formylpyridine contained an ethyl urea moiety. In the synthesis
 140 of **8**, propylamine was reacted with 5-nitro-2-formylpyridine, and the nitro/imine
 141 intermediate **8a** was reduced to the amine/amine ligand **8b**. Complexation to obtain
 142 complexes **6-8** was achieved with ZnSO₄. In the case of **9**, the zwitterionic amine taurine was
 143 reacted with 2-formylpyridine under basic conditions, followed by complexation with ZnCl₂
 144 in the same pot. While this sub-set of molecules also offers extensive H-bonding patterns,
 145 they have larger steric requirements compared with some of the asymmetric members of the
 146 series.

148 **Scheme 3.** Synthesis of pyridino zinc(II) complexes **6-9** (yields: **6**, 83%; **7**, 51 %; **8**, 30%; **9**, 20%)

Hydroxamic complexes **10** and **11** were generated as shown in Scheme 4. A benzoic acid functionalized at the 4-position with an amine handle (either directly attached to the ring or as an aminomethylene moiety respectively) was converted to its corresponding methyl ester using known procedures.^[16] A critical step in ligand preparation was the transformation of these esters to hydroxamic acids using hydroxylamine sulfate under basic aqueous conditions. Conversion of methyl ester **10a** to hydroxamic acid **10b** was performed in one step^[17] leaving the free amine intact, while **11a** underwent modification with Tf₂O to form sulphonamide **11b**, prior to its conversion to the desired hydroxamic acid **11c**. Ligands **10b** and **11c** afforded (2:1 ligand-to-metal) complexes upon reaction with Zn(OAc)₂. Hydroxamates are considered excellent chelators for metal ion transport through cell membranes,^[18] while both the hydroxamate^[18] and sulphonamide^[19] functionalities are of high medicinal relevance and present in many bioactive compounds.

Scheme 4. Synthesis of hydroxamic zinc(II) complexes **10-11** (yields: **10**, 92%; **11**, 84%)

166

167 **Biological evaluation of the effect of compounds on the Insulin/PI3K/PTEN/Akt**
168 **pathway.**

169 **Zinc complexes induce Akt activation**

170 The zinc(II) ion and some of its complexes have been reported in the past to elicit activation
171 of Akt in several cell types.^[11a, 20] The activation of Akt in these cases occurs via the PI3K
172 route and can be monitored by detecting the level of phosphorylation of residue Thr-308 or
173 Ser-473 of Akt. To examine whether the new zinc complexes prepared in this study induce
174 Akt activation, NIH3T3 cells were treated with 10 μ M of each compound, followed by light
175 stimulation with 0.04 μ M insulin. Western blots were run from the cell lysates and phospho-
176 Akt was quantified using a phospho-Akt (Ser473) antibody. Total Akt was also quantified as
177 loading control. Figure 3 (A and B) summarizes these results. A positive control (stimulation
178 with high insulin, 2 μ M) and a negative control (low insulin equal to the concentration used
179 to activate the insulin receptor, 0.04 μ M) were included for comparison. All compounds, with
180 the exception of **1-3** whose effect is negligible, appear to activate Akt by elevating phospho-
181 Akt levels to different extents. While the majority of the compounds exhibit Akt activation to
182 around 50% of the positive control, the two compounds derived from hydroxamic acid
183 ligands (**10** and **11**) stand out, as they appear to perform equally effective or considerably
184 better than the high insulin activation control respectively.

Figure 3. A (compounds **1-5**) and B (compounds **6-11**): NIH3T3 cells were starved overnight, incubated with 10 μ M of compound (or vehicle) for 15 min, followed by insulin stimulation as indicated. Cells were collected and analyzed by western blotting using phospho-Akt (Serine-473) and Akt antibodies. The blots in Figures 6A and 6B were quantified using ImageJ. The percentage of activation of Akt (p-Akt phosphorylation normalized for loading using the Akt blotting intensities) is shown. Low insulin stimulation (0.04 μ M) was set to 0% and high insulin stimulation (2 μ M) was set to 100%, respectively. Results (bar chart) are presented as the mean $\pm$ standard deviation of three independent experiments.

Compound **11**, as the most promising Akt-activator, was subjected to further investigation. First, we determined the cytotoxicity of this compound with the MTT assay on NIH3T3 cells.^[21] MTT is reduced to formazan by living cells which can be quantified using a spectrophotometer at an absorbance of 590 nm. As can be seen in Figure 4, compound **11** shows cytotoxicity effects on NIH3T3 cells at concentrations equal or higher than 100 μ M.

At the concentration of 10 μ M, which were used in the initial screen of all 11 compounds (Figure 3), compound **11** has no cytotoxic effect on the cells.

Figure 4 Cytotoxicity of compound **11**. NIH3T3 cells were treated with up to 1 mM of compound **11**. The results are presented as mean $\pm$ standard deviations ($n = 4$).

To determine the minimum concentration of compound **11** required to induce Akt activation, NIH3T3 cells were treated with various concentrations of the complex. As can be seen in Figure 5A, Akt activation commenced at a concentration of 500 nM of compound **11**. Moreover, when probing the compound-treated cell lysates for glucogen synthase kinase 3 β (GSK-3 β) phosphorylation at Ser-9, we found that **11** was also able to stimulate GSK-3 β phosphorylation with similar potency (Figure 5A). GSK-3 β , a key enzyme in the regulation of glucose metabolism, is phosphorylated at Ser-9 by Akt, suggesting that the activation of Akt induced by the Zn complexes is propagated downstream of Akt to enhance glycogen synthesis. For comparison, the effect of compound **11** on the MAP kinase pathway was investigated by probing cellular extracts for phosphorylation of p42 and p 44 MAP kinases. As shown in Figure 5B, compound **11** fails to activate MAPK p44/p42 at a concentration of

500 nM implying that compound **11** might be a useful chemical tool to probe glucose metabolism and the signalling controlling it.

Figure 5. (A) Activation of Akt and downstream element GSK-3 β (Serine-9) with compound **11** at various concentrations. Starved cells were incubated with 100 nM, 250 nM and 500 nM of compound **11** for 15 min, followed by stimulation of insulin at a concentration of 0.04 μ M. (B) Compound **11** does not increase phosphorylation levels of p44/p42 MAPK. Same cell lysates (from figure 5A) were probed with a phospho-p44/42 MAPK (Thr202/Tyr204) antibody.

Zinc complex-induced Akt activation is PI-3kinase dependent

To investigate whether the observed Akt phosphorylation is PI3K-mediated, we studied the effects of compound **11** on Akt phosphorylation levels in the presence of LY-294002, a known PI3K inhibitor.^[22] If the observed Akt activation is PI3K dependent, the presence of LY-294002 would be expected to abolish the activation induced by the zinc compound. Indeed, use of LY-294002 abrogated the compound **11**-induced Akt phosphorylation as shown in Figure 6. To further confirm the hypothesis that the zinc complex-mediated activation occurs via PI3K, we examined whether any of the prepared complexes can induce Akt activation without any insulin stimulation. Notably, when insulin is completely deprived,

the compounds are unable to induce Akt phosphorylation (Figure 7), which suggests they do not act directly at the insulin receptor level, but rather between receptor and PI3K. Taking these observations together, we suggest that the zinc compounds induce Akt activation in a PI3K-dependent fashion.

Figure 6. Effects of compound **11** on Akt phosphorylation with and without the PI3K inhibitor LY294002.

Figure 7. Phospho-Akt (Serine-473) blots after cells were treated either with the zinc complexes without any insulin stimulation or with insulin (2 μ M). Loading control is performed using the detection of total Akt.

Zinc complex-induced Akt activation is not via inhibition of PTPases or PTEN

The PI3K/Akt cascade is modulated by phosphatases of the PTPase family and PTEN. PTPases oppose the signalling mediated by receptor tyrosine kinases by removing tyrosine phosphorylation, whereas PTEN directly antagonises PI3K by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3), the product of PI3K activity. As both PTPases and PTEN are upstream of Akt, their phosphatase activity can influence the level of Akt activation. Indeed, it has been found that inhibition of PTPases and PTEN will increase the cellular activity of Akt.^[23]

Zinc(II) has been reported to have the ability to inhibit phosphatases such as λ -phosphoprotein phosphatase,^[24] protein tyrosine phosphatase 1B,^[25] as well as PTEN.^[11b] In order to investigate whether the observed Akt activation was due to inhibition of PTEN and PTPases, the two most promising Akt-activating compounds (hydroxamic subset) **10** and **11** were evaluated as PTEN and PTP- β inhibitors *in vitro*. As shown in Figure 8 the inhibition of PTP- β and PTEN by compound **10** and **11** revealed a significantly higher potency towards the PTP- β , but failed to demonstrate the nanomolar potency achieved for Akt phosphorylation *in cellulo*. A similar picture was observed for other representative members of the series (results not shown).

Figure 8. Inhibition of PTEN and PTP- β with zinc(II) complexes **10** and **11** using OMFP and *p*-NPP as substrates. Recombinant PTEN and PTP- β were pre-incubated with either compound **10** or **11** at various concentrations at room temperature. Reactions were initialized by adding 200 μ M of OMFP for PTEN and 1 mM of *p*-NPP for PTP- β . The IC₅₀ in the presence of compound **10** was calculated to be 116 ± 25 μ M for PTEN and 42 ± 7 μ M for PTP- β . The IC₅₀ of compound **11** was determined to be 77 ± 5 μ M for PTEN and 19 ± 1 μ M for PTP- β . The results are presented as the mean $\pm$ the standard deviation of three independent experiments.

Conclusion

In summary, a series of eleven novel zinc(II) complexes incorporating medicinally-relevant functionalities were prepared based on phenolic, picolinic, pyridino and hydroxamic ligands. Screening of the compounds for their potential to upregulate Akt phosphorylation *in cellulo*, revealed a sulphonamide-containing hydroxamic complex as a potent activator of Akt at sub-micromolar concentrations. This lead structure can be potentially used as a chemical tool in diabetes research, due to the fact that apart from activating Akt signalling, its effect is propagated downstream of Akt towards GSK-3 β , an important regulator of glycogen synthesis/glucose metabolism. Our findings suggest that the observed effects on Akt phosphorylation by the hydroxamic zinc complexes, while PI3K-mediated, are not due to the

inhibition of the tumor-suppressor phosphatase PTEN or PTPases. This signifies a departure from other zinc and vanadium complexes that have been shown to cause Akt activation by inhibiting these phosphatases.

Future research will be directed towards elucidating the exact mechanism of action of hydroxamic zinc complexes on Akt activation, as well as the development of improved Akt activators based on evolution of this novel template.

Experimental Section

General Procedures

The purity of the organic chemicals was verified by ¹H-NMR spectroscopy. ¹H-NMR and ¹³C-NMR spectra were recorded on a Bruker Avance 400 MHz Ultrashield NMR spectrometer. Infra-red spectra were recorded on a Perkin-Elmer FTIR spectrometer. Electrospray ionisation mass spectra were recorded on a Bruker Daltronics Esquire 3000 spectrometer. Elemental analyses were performed by either London Metropolitan University or University of Cambridge. The following compounds were prepared following literature procedures: **1a**,^[12] **5a**,^[15] **10a**,^[16a] **10b**^[17] and **11a**.^[16b]

Synthesis

Synthesis of 1

Intermediate **1a** (0.107 g, 0.35 mmol, 1 equiv) was suspended in 45 mL of methanol. Ethanolamine (42 μ L, 0.70 mmol, 2 equiv) was added and the mixture was stirred at r.t. overnight. The resulting solution was then cooled at 0°C and 2 mL of a methanolic solution

of NaBH₄ (0.026 g, 0.70 mmol, 2 equiv) was added dropwise. The mixture was stirred initially at 0°C for 1 h, then allowed to warm up to r.t. It was then filtered and the collected yellow precipitate was washed with methanol and dried to afford 43% of imine intermediate (0.053 g, 0.15 mmol). This compound was then dissolved in 10 mL of water to afford a clear solution. Aqueous HCl 0.6 M (0.25 mL, 0.15 mmol, 1 equiv) was added to the solution and the mixture stirred at r.t. briefly, before evaporating the solvent to dryness. The obtained solid residue was washed with diethyl ether to afford complex **1** in nearly quantitative yield (0.058 g, 0.15 mmol). ¹H-NMR (D₂O, 4.79 ppm)-δ (ppm): 3.07 (t, 2H, *J*=5.4 Hz), 3.75 (t, 2H, *J*=5.4 Hz), 4.06 (s, 2H), 4.08 (s, 2H), 6.69 (bd, 1H, *J*=8.6 Hz), 7.24 (m, 2H), 8.26 (s, 1H). ¹³C-NMR (D₂O)-δ (ppm): 48.1, 50.4, 56.7, 56.8, 119.5, 122.4, 126.0, 135.2, 137.6, 149.9, 168.9. Elemental analysis: calculated for C₁₂H₁₉ClN₂O₆Zn: C 37.13, H 4.93, N 7.22; found: C 36.57, H 4.12, N 6.96.

Synthesis of 2

Racemic homocysteic acid (0.150 g, 0.82 mmol, 1 equiv) was added to 5 mL of water and the resulting mixture was heated at 70°C. A solution of 3-formyl-4-hydroxy-benzenesulfonic acid disodium salt (0.201 g, 0.82 mmol, 1 equiv) in 5 mL of water was then added and the mixture was stirred at 70 °C for 90 mins. A solution of Zn(OAc)₂·2H₂O (0.223 g, 1.02 mmol, 1.24 equiv) in 10 mL of water was finally added. During this last addition the pH of the solution was adjusted to about 7 with aqueous NaOH 0.5 M. The solution was stirred at 70°C for two more hours. The solvent was then evaporated to dryness and a yellow-orange residue was obtained. Successive recrystallizations in water/methanol yielded compound **2** as a white solid in 54% yield (0.217 g, 0.44 mmol). ¹H-NMR (D₂O, 4.79 ppm)-δ (ppm): 2.25 (m, 2H), 2.86 (m, 2H), 4.03 (t, 1H, *J*=6.2 Hz), 6.72 (d, 1H, *J*₁=9.0 Hz), 7.57 (dd, 1H, *J*₁=9.0 Hz, *J*₂=2.5 Hz), 7.66 (d, 1H, *J*₂=2.5 Hz), 8.38 (s, 1H). ¹³C-NMR (D₂O)-δ (ppm): 29.9, 46.8, 66.3,

118.4, 122.2, 128.5, 130.9, 133.9, 169.7, 171.1, 179.1. Elemental Analysis: calculated for $C_{11}H_{13}NNa_2O_{11}S_2Zn \cdot 2H_2O$: C 24.16, H 3.16, N 2.56; found C 24.42, H 3.33, N 2.54.

Synthesis of 3a

3-Formyl-4-hydroxy-benzenesulfonic acid disodium salt (0.300g, 1.22 mmol, 1 equiv) was dissolved in 40 mL of methanol, and propylamine (0.121 mL, 1.47 mmol, 1.2 equiv) was added. The solution was stirred at r.t. for 3 h, at which point an aliquot was obtained to verify by NMR that the imine intermediate had formed. The solution was then cooled in an ice-bath and $NaBH_4$ (0.185 g, 4.88 mmol, 4 equiv) was added. The mixture was stirred at 0°C for 1 h and at r.t. overnight. A few drops of water were added and then the solvent was evaporated to dryness. The residue was re-dissolved in methanol and insoluble impurities were removed by filtration. The filtrate was concentrated under vacuum and diethyl ether was added to precipitate the product. Compound **3a** was obtained as a pink solid in 72% yield (0.255 g, 0.88 mmol). 1H -NMR (D_2O , 4.79 ppm)- δ (ppm): 0.85 (t, 3H, $J=7.4$ Hz), 1.58 (bs, 2H), 2.88 (t, 2H, $J=7.4$ Hz), 4.02 (s, 2H), 6.55 (d, 1H, $J=8.6$ Hz), 7.45 (m, 2H). This ligand was used for the synthesis of **3** without any further purification.

Synthesis of 3

To a solution of **3a** (0.100 g, 0.35 mmol, 1 equiv) in 10 mL of methanol was added a solution of $Zn(OAc)_2 \cdot 2H_2O$ (0.038 g, 0.175 mmol, 0.5 equiv) in 5 mL of methanol and the resulting mixture was stirred at 50 °C overnight. The solvent was evaporated to dryness and complex **3** was obtained as a yellow residue. It was further purified by recrystallization in methanol/diethyl ether. 1H -NMR (D_2O , 4.79 ppm)- δ (ppm): 0.86 (t, 6H, $J=7.1$ Hz), 1.59 (bs, 4H), 2.90 (bt, 4H, $J=6.7$ Hz), 4.06 (s, 4H), 6.63 (d, 2H, $J=9.2$ Hz), 7.59 (m, 4H). Also

present: 1 equiv. of NaOAc impurity: 1.82 (s, 3H). Elemental Analysis: calculated for $C_{20}H_{26}N_2Na_2O_8S_2Zn \cdot NaOAc$: C 38.86, H 4.30, N 4.12; found: C 38.77, H 4.41, N 3.98.

Synthesis of 4

A solution of ethylenediamine (0.027 mL, 0.41 mmol, 0.5 equiv) in 2 mL of methanol was added to a solution of 3-formyl-4-hydroxy-benzenesulfonic acid disodium salt (0.202 g, 0.82 mmol, 1 equiv) in 40 mL of methanol and the reaction mixture was stirred at r.t for 1 h. $Zn(OAc)_2 \cdot 2H_2O$ (0.090 g, 0.41 mmol, 0.5 equiv) was then added and the mixture was heated at 50°C overnight. It was subsequently filtered, and the collected white residue was washed with cold methanol and dried under vacuum to afford **4** in 95% yield (0.216 g, 0.39 mmol). 1H -NMR (D_2O , 4.79 ppm)- δ (ppm): 3.79 (s, 4H), 6.80 (d, 2H, $J_1=8.9$ Hz), 7.55 (dd, 2H, $J_1=8.9$ Hz, $J_2=2.5$ Hz), 7.64 (d, 2H, $J_2=2.5$ Hz), 8.43 (s, 2H). ^{13}C -NMR (D_2O)- δ (ppm): 55.8, 119.2, 122.6, 128.3, 130.0, 133.1, 168.1, 170.8. Elemental Analysis: calculated for $C_{16}H_{14}N_2Na_2O_9S_2Zn \cdot 0.5H_2O$: C 34.15, H 2.69, N 4.98; found: C 34.11, H 2.72, N 4.89.

Synthesis of 5

A solution of $ZnSO_4 \cdot 7H_2O$ (0.050 g, 0.175 mmol, 0.5 equiv) in 1.5 mL of water was added to a solution of **5a** (0.080 g, 0.35 mmol, 1 equiv) in 3 mL of EtOH/DMF (1:1). Aqueous NaOH 0.5 M (0.7 mL, 0.35 mmol, 1 equiv) was added and the mixture was stirred at r.t. for 3 h. The resulting precipitate was then filtered off, washed with water and dried under vacuum to furnish 34% of complex **5** (0.033 g, 0.06 mmol). 1H -NMR ($DMSO-d_6$, 2.50 ppm)- δ (ppm): 4.34 (d, 4H, $J=5.2$ Hz), 7.12 (d, 2H, $J=7.2$ Hz), 7.24 (m, 2H), 7.32 (m, 8H), 7.45 (bs, 2H), 7.85 (d, 2H, $J=8.8$ Hz), 7.94 (bs, 2H). Elemental Analysis: calculated for $C_{26}H_{26}N_4O_6Zn$: C 56.17, H 4.71, N 10.08; found: C 56.18, H 4.60, N 10.14.

Synthesis of **6a**

Ethyl isocyanate (0.29 mL, 3.66 mmol, 1 equiv) was slowly added to a cooled solution (−10 °C) of 1,3-diaminopropane (0.305 mL, 3.66 mmol, 1 equiv) in 35 mL of CH₂Cl₂. The mixture was then allowed to stir overnight at r.t., resulting in precipitation of the desired urea product (**6a**). This solid was collected by filtration. Additionally, the filtrate was dried under vacuum to yield an oily residue, which upon treatment with diethyl ether afforded more of the product. The combined product was washed with diethyl ether and dried. Overall, 63% of **6a** was obtained (0.335 g, 2.31 mmol). ¹H-NMR (DMSO-d₆, 2.50 ppm)-δ (ppm): 0.95 (t, 3H, *J*=7.0 Hz), 1.40 (m, 2H), 2.50 (m, 2H), 3.05 (m, 4H), 5.80 (bs, 2H). LC-MS (ESI⁺): calculated for C₆H₁₅N₃O (M+H) 146.13, found 146.13. This compound was used in the next step without any further purification.

Synthesis of **6b**

Pyridine-2-carboxaldehyde (0.10 mL, 1.0 mmol, 1 equiv) was slowly added (over 10 min) to a cold solution (−10 °C) of **6a** (0.145 g, 1.0 mmol, 1 equiv) in 2 mL of methanol, and the mixture was allowed to stir overnight at r.t. The solvent was then removed under vacuum to yield a white residue, which was washed several times with diethyl ether and dried to furnish 93% of **6b** (0.217 g, 0.93 mmol). ¹H-NMR (DMSO-d₆, 2.50 ppm)-δ (ppm): 0.97 (t, 3H, *J*=7.2 Hz), 1.73 (m, 2H), 2.98 (m, 2H), 3.05 (apparent q, 2H, *J*=6.4 Hz), 3.62 (t, 2H, *J*=6.8 Hz), 5.76 (t, 1H, *J*=5.6 Hz), 5.86 (t, 1H, *J*=5.6 Hz), 7.45 (m, 1H), 7.86 (td, 1H, *J*₁=7.6 Hz, *J*₂=1.2 Hz), 8.34 (s, 1H), 8.63 (d, 1H, *J*=4.8 Hz), 8.94 (d, 1H, *J*₁=7.6 Hz). ¹³C-NMR (DMSO-d₆, 39.52 ppm)-δ (ppm): 16.2, 31.6, 34.5, 37.7, 58.4, 120.9, 125.5, 137.3, 149.8, 154.6, 158.5, 162.4. Elemental Analysis: calculated for C₁₂H₁₈N₄O: C 61.52, H 7.74, N 23.91; found: C 61.52, H 7.64, N 23.84.

Synthesis of **6**

A solution of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.100 g, 0.35 mmol, 0.5 equiv) in 2 mL of water was added to a solution of **6b** (0.163 g, 0.70 mmol, 1 equiv) in 2 mL of methanol, and the resulting mixture was stirred at r.t. for 2 h. The solvent was then removed under vacuum, yielding complex **6** as a pale brown solid in 83% yield (0.193 g, 0.29 mmol). ^1H -NMR (DMSO-d_6 , 2.50 ppm)- δ (ppm): 0.88 (t, 6H, $J=7.2$ Hz), 1.74 (m, 4H), 2.86 (m, 4H), 3.01 (apparent q, 4H, $J=5.6$ Hz), 3.65 (bs, 4H), 5.85 (bs, 2H), 6.17 (bs, 2H), 7.66 (bs, 2H), 7.94 (d, 2H, $J=7.6$ Hz), 8.08 (bs, 2H), 8.52 (s, 2H), 8.78 (bs, 2H). Elemental Analysis: calculated for $\text{C}_{24}\text{H}_{40}\text{N}_8\text{O}_8\text{SZn} \cdot \text{H}_2\text{O}$: C 42.14, H 6.19, N 16.38; found: C 42.33, H 5.69, N 16.33.

Synthesis of **7a**

This compound was prepared in 28% yield, following an analogous method to the one used for the synthesis of **6a**. ^1H -NMR (DMSO-d_6 , 2.50 ppm)- δ (ppm): 0.95 (t, 3H, $J=7.1$ Hz), 1.40 (m, 4H), 2.50 (bt, 2H, overlapping with DMSO peak), 3.00 (m, 4H), 5.71 (bt, 1H), 5.80 (bt, 1H). LC-MS (ESI^+): calculated for $\text{C}_7\text{H}_{17}\text{N}_3\text{O}$ ($\text{M}+\text{H}$) 160.15, found 160.15. This compound was used in the next step without any further purification.

Synthesis of **7b**

This compound was prepared in 99% yield, following an analogous method to the one used for the synthesis of **6b**. ^1H -NMR (DMSO-d_6 , 2.50 ppm)- δ (ppm): 0.96 (t, 3H, $J=7.2$ Hz), 1.40 (m, 2H), 1.61 (m, 2H), 2.99 (m, 4H), 3.62 (t, 2H, $J=7.2$ Hz), 5.71 (t, 1H, $J=5.6$ Hz), 5.80 (t, 1H, $J=5.2$ Hz), 7.45 (m, 1H), 7.86 (td, 1H, $J_1=7.6$ Hz, $J_2=1.2$ Hz), 7.93 (d, 1H, $J_1=7.6$ Hz), 8.33 (s, 1H), 8.63 (d, 1H, $J=4.8$ Hz). ^{13}C -NMR (DMSO-d_6 , 39.52 ppm)- δ (ppm): 16.2, 28.2, 28.4, 34.5, 60.6, 120.8, 125.5, 137.3, 149.8, 154.6, 158.5, 162.1. Elemental Analysis: calculated for $\text{C}_{13}\text{H}_{20}\text{N}_4\text{O}$: C 62.88, H 8.12, N 22.56; found: C 62.96, H 8.09, N 22.48.

Synthesis of **7**

This compound was prepared in 51% yield, following an analogous method to the one used for the synthesis of **6**. ¹H-NMR (DMSO-d₆, 2.50 ppm)-δ (ppm): 0.93 (t, 6H, *J*=7.2 Hz), 1.31 (m, 4H), 1.61 (m, 4H), 2.95 (m, 8H), 3.60 (bs, 4H), 5.93 (bs, 2H), 6.02 (bs, 2H), 7.67 (bs, 2H), 7.97 (d, 2H, *J*=7.6 Hz), 8.10 (bs, 2H), 8.53 (s, 2H), 8.82 (bs, 2H). Elemental Analysis: calculated for C₂₆H₄₄N₈O₈SZn·2.5H₂O: C 42.25, H 6.68, N 15.16; found: C 42.13, H 5.89, N 15.16.

Synthesis of **8a**

A solution of 5-nitropyridine-2-carboxaldehyde (0.386 g, 2.54 mmol, 1 equiv) in 20 mL of dry methanol was added dropwise to a solution of propylamine (0.24 mL, 2.92 mmol, 1.15 equiv) in 8 mL of methanol. The mixture was stirred for 5 h at r.t. and the solvent was then removed under vacuum, to afford **8a** quantitatively as a dark oil (0.49 g, 2.54 mmol). ¹H-NMR (CDCl₃, 7.26 ppm)-δ (ppm): 1.00 (t, 3H, *J*=7.4 Hz), 1.79 (apparent sextet, 2H, *J*=7.2 Hz), 3.73 (td, 2H, *J*₁=6.9 Hz, *J*₂=1.3 Hz), 8.23 (d, 1H, *J*₃=8.7 Hz), 8.47 (bs, 1H), 8.53 (dd, 1H, *J*₃=8.7 Hz, *J*₄=2.4 Hz), 9.47 (d, 1H, *J*₄=2.4 Hz). ¹³C-NMR (CDCl₃, 77.00 ppm)-δ (ppm): 11.9, 23.8, 63.5, 121.21, 131.6, 144.4, 144.9, 159.2, 159.9. This compound was used for the next step without any further purification.

Synthesis of **8b**

A solution of **8a** (0.293 g, 1.52 mmol, 1 equiv) in 25 mL of dry methanol was mixed with 0.09 g of 10% Pd-C and cooled at 5°C. NaBH₄ (0.23 g, 6.1 mmol, 4 equiv) was added portionwise to the mixture over 30 min. The resulting solution was stirred at the same temperature for 1 h, then allowed to warm up to r.t. It was subsequently filtered through celite

and the solvent was evaporated to dryness. The remaining residue was re-suspended in ethyl acetate and the organic phase was washed with water. The aqueous phase was back-extracted with CHCl_3 . All organic fractions were dried over MgSO_4 and the solvent was evaporated to dryness to afford **8b** as a yellow oil in 55% yield (0.139 g, 0.84 mmol). ^1H -NMR (CDCl_3 , 7.26 ppm)- δ (ppm): 0.93 (m, 3H), 1.54 (m, 2H), 2.61 (m, 2H), 3.66 (bs, 2H), 3.79 (s, 2H), 6.96 (dd, 1H, $J_1=8.2$ Hz, $J_2=2.8$ Hz), 7.09 (d, 1H, $J_1=8.2$ Hz), 8.06 (d, 1H, $J_2=2.8$ Hz). ^1H -NMR (DMSO-d_6 , 2.50 ppm)- δ (ppm): 0.85 (m, 3H), 1.41 (m, 2H), 2.46 (m, 2H), 3.60 (s, 2H), 5.13 (bs, 2H), 6.88 (dd, 1H, $J_1'=8.3$ Hz, $J_2'=2.7$ Hz), 7.03 (d, 1H, $J_1'=8.3$ Hz), 7.85 (d, 1H, $J_2'=2.7$ Hz). ^{13}C -NMR (CDCl_3 , 77.00 ppm)- δ (ppm): 11.8, 23.2, 51.5, 54.7, 122.3, 122.6, 136.9, 141.0, 149.9. ^{13}C -NMR (DMSO-d_6 , 39.52 ppm)- δ (ppm): 12.3, 22.9, 51.1, 54.4, 121.0, 122.4, 135.8, 143.7, 147.3. This compound was used for the next step without any further purification.

Synthesis of 8

A solution of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.121g, 0.42 mmol, 0.5 equiv) in 10 mL of methanol was added to a solution of **8b** (0.139 g, 0.84 mmol, 1 equiv) in 10 mL of methanol, and the resulting mixture was stirred at r.t. for 3 h. The solvent was then evaporated to dryness and the residue washed with dichloromethane and ethyl ether. Recrystallization in methanol/diethyl ether afforded complex **8** as a yellow solid in 30% yield (0.066 g, 0.125 mmol). ^1H -NMR (DMSO-d_6 , 2.50 ppm)- δ (ppm): 0.81 (t, 6H, $J=7.3$ Hz), 1.50 (m, 4H), 2.62 (bs, 4H), 3.80 (bs, 4H), 5.58 (bs, 2H), 5.71 (bs, 4H), 7.18 (bs, 4H), 8.03 (bs, 2H). ^{13}C -NMR (DMSO-d_6 , 39.52 ppm)- δ (ppm): 11.8, 20.9, 50.3, 50.4, 123.3, 123.7, 134.7, 142.4, 145.6. LC-MS (ESI^+): calculated for $\text{C}_{18}\text{H}_{28}\text{N}_6\text{Zn}$ ($\text{M}+\text{H}$) 393.18, found 393.18. IR (cm^{-1}): 1118 (SO_4^{2-}), 1506 (m), 1581 (m), 1630 (m), 1735 (br), 2875 (br), 2964 (br), 3229, 3349. Elemental Analysis: calculated for $\text{C}_{18}\text{H}_{34}\text{N}_6\text{O}_6\text{SZn} \cdot 2\text{H}_2\text{O}$: C 38.33, H 6.79, N 14.90; found: C 38.58, H 6.15, N 14.27.

506

507 *Synthesis of 9*

508 Pyridine-2-carboxaldehyde (0.19 mL, 2 mmol, 1 equiv) was dissolved in 50 mL DMF in a
509 round-bottom flask. Taurine (0.25 g, 2 mmol, 1 equiv) was added to this solution, followed
510 by anhydrous triethylamine (0.28 mL, 2 mmol, 1 equiv). After stirring at r.t. until all
511 components had gone into solution, ZnCl₂ was added (0.14 g, 1 mmol, 0.5 equiv) and the
512 reaction mixture was heated at 110°C for 4h. The resulting precipitate was filtered and
513 washed to afford **9** as a pink powder in 20% yield (0.10 g, 0.2 mmol). ¹H-NMR (DMSO-d₆,
514 2.50 ppm)-δ (ppm): 3.03 (bs, 4H), 4.24 (bs, 4H), 7.67 (t, 2H, *J*=6.4 Hz), 8.03 (d, 2H, *J*=8.0
515 Hz), 8.17 (bs, 2H), 8.36 (bs, 2H), 8.88 (bs, 2H). ¹³C-NMR (DMSO-d₆, 39.52 ppm)-δ (ppm):
516 49.86, 54.46, 128.94, 141.11, 148.63, 163.60. IR (cm⁻¹): 738, 1228, 1530, 1594, 2943, 3235,
517 3331. Elemental analysis: calculated for C₁₆H₁₈N₄O₆S₂Zn: C 39.07, H 3.69, N 11.39; found:
518 C 39.13, H 3.73, N 11.45.

519

520 *Synthesis of 10*

521 4-Amino-*N*-hydroxybenzamide (**10b**) (0.20 g, 1.32 mmol, 1 equiv) was transferred to a
522 round-bottom flask and dissolved in methanol (13.2 mL). Zn(OAc)₂·2H₂O (0.145 g, 0.66
523 mmol, 0.5 equiv) was added, and the reaction was refluxed at 65°C for 12 h, during which a
524 white precipitate formed. After cooling at r.t., the mixture was filtered and the solid washed
525 several times with chilled water and dried under vacuum. Overall, 92% (0.235 g, 0.61 mmol)
526 of complex **10** was obtained. ¹H-NMR (DMSO-d₆, 2.50 ppm)-δ (ppm): 5.50 (s, 4H), 6.53 (d,
527 4H, *J*=8.6 Hz), 7.48 (d, 4H, *J*=8.6 Hz), 11.50 (s, 2H). ¹H-NMR (D₂O, 4.79 ppm, with 1%
528 formic acid-d₂)-δ (ppm): 7.26 (d, 4H, *J*=8.0 Hz), 7.71 (d, 4H, *J*=8.0 Hz). LC-MS (ESI⁺):
529 calculated for C₁₄H₁₄N₄O₄Zn (M+H) 367.04, found 367.04. IR (cm⁻¹): 1504, 1563, 1598,

3018, 3170, 3431. Elemental analysis: calculated for $C_{14}H_{16}N_4O_5Zn$: C 43.59, H 4.18, N 14.53; found: C 44.02, H 3.71, N 14.35.

Synthesis of 11b

Methyl 4-(aminomethyl)benzoate (**11a**) (1.65 g, 10 mmol, 1 equiv) was transferred to a round-bottom flask, which was then sealed and purged with nitrogen. Anhydrous CH_2Cl_2 (33 mL) was added and the mixture was cooled at $-78^\circ C$. Triethylamine (2.8 mL, 20 mmol, 2 equiv) was added, followed by dropwise addition of trifluoromethanesulfonic anhydride (1.7 mL, 10 mmol, 1 equiv) at $-78^\circ C$. The mixture was allowed to stir over 3-4 h, with the temperature slowly rising to $0^\circ C$. The reaction mixture was then diluted with CH_2Cl_2 , and washed once with aqueous NH_4Cl (sat), once with aqueous $NaCl$ (sat) and once with H_2O . The organic phase was dried over Na_2SO_4 , then filtered and concentrated under vacuum. The sample was applied to a silica column and eluted with hexane-ethyl acetate (first 2:1, then 1:1), to afford **11b** in 88% yield (2.61 g, 8.8 mmol) as a white solid. 1H -NMR ($CDCl_3$, 7.26 ppm)- δ (ppm): 3.92 (s, 3H), 4.51 (s, 2H), 5.43 (bs, 1H), 7.39 (d, 2H, $J=8.3$ Hz), 8.01 (d, 2H, $J=8.3$ Hz). ^{13}C -NMR ($CDCl_3$, 77.00 ppm)- δ (ppm): 47.65, 52.37, 119.62 (CF_3 quartet), 127.61, 130.03, 130.19, 140.49, 166.89. LC-MS (ESI^+): calculated for $C_{10}H_{10}F_3NO_4S$ (M+H) 298.04, found 298.04. IR (cm^{-1}): 1001, 1018, 1081, 1119, 1150, 1181, 1233, 1289, 1306, 1386, 1445, 1615, 1695, 3229. Elemental analysis: calculated for $C_{10}H_{10}F_3NO_4S$: C 40.41, H 3.39, N 4.71; found C 40.51, H 3.27, N 4.64.

Synthesis of 11c

Hydroxylamine sulfate (0.82 g, 5 mmol, 1 equiv) was transferred to a round-bottom flask containing 5 g of ice. A solution of NaOH (1.0 g, 25 mmol, 5 equiv) in 5 mL of water was added to the flask and the mixture was stirred at r.t. for 5 min. A catalytic amount of Na_2SO_3

(0.095 g, 0.75 mmol, 0.15 equiv) and methyl 4-[(trifluoromethylsulfonamido)methyl]-benzoate (**11b**) (1.49 g, 5 mmol, 1 equiv) were then added in solid form, and the mixture was heated at 45°C for 16 h. After cooling at r.t., the pH was modified to about 5 by dropwise addition of aqueous H₂SO₄ 10% (w/w), and the solvent was removed under vacuum. Cold methanol was added to the solid residue and the resulting suspension was vigorously stirred for 15 mins and filtered. The hydroxamic acid, free of most inorganic impurities, was recovered by drying the methanolic filtrate. This sample was re-dissolved in CH₂Cl₂-CH₃OH 90:10 and applied to a silica column. Elution took place with the same solvent system (first 90:10, then 85:15) to afford 76% (1.13 g, 3.8 mmol) of hydroxamic acid **11c** as a white solid. ¹H-NMR (DMSO-d₆, 2.50 ppm)-δ (ppm): 4.42 (s, 2H), 7.40 (d, 2H, *J*=8.1 Hz), 7.75 (d, 2H, *J*=8.1 Hz), 9.04 (s, 1H), 10.02 (s, 1H), 11.22 (s, 1H). ¹³C-NMR (DMSO-d₆, 39.52 ppm)-δ (ppm): 46.34, 119.69 (CF₃ quartet), 127.21, 127.38, 132.20, 140.22, 163.92. LC-MS (ESI⁺): calculated for C₉H₉F₃N₂O₄S (M+H) 299.03, found 299.03. IR (cm⁻¹): 1511, 1542, 1577, 1618, 3388. Elemental analysis: calculated for C₉H₉F₃N₂O₄S: C 36.24, H 3.04, N 9.39; found C 36.25, H 3.02, N 9.11.

Synthesis of 11

N-hydroxy-4-[(trifluoromethylsulfonamido)methyl]benzamide (0.63 g, 2.11 mmol, 1 equiv) was transferred to a round-bottom flask and dissolved in absolute ethanol (15 mL). A solution of NaOH (0.084 g, 2.11 mmol, 1 equiv) in 5 mL of water was added, and the mixture was stirred at r.t. for 5 min. Solid Zn(OAc)₂·2H₂O (0.23 g, 1.055 mmol, 0.5 equiv) was then added, and the reaction was refluxed at 80°C for 16 h. The solvent was then removed under vacuum and the solid residue washed extensively with cold water and dried under vacuum to afford complex **11** in 84% yield (0.60 g, 0.89 mmol), as a white solid. ¹H-NMR (DMSO-d₆, 2.50 ppm)-δ (ppm): 4.27 (s, 4H), 7.35 (d, 4H, *J*=7.7 Hz), 7.66 (d, 4H, *J*=7.7 Hz), 11.82 (s,

2H). LC-MS (ESI⁺): calculated for C₁₈H₁₆F₆N₄O₈S₂Zn (M+H) 658.97, found 658.97. IR (cm⁻¹): 874, 918, 1022, 1056, 1149, 1190, 1231, 1372, 1441, 1503, 1565, 1606, 3350 (br). Elemental analysis: calculated for C₁₈H₁₈F₆N₄O₉S₂Zn.H₂O: C 31.07, H 2.90, N 8.05; found C 31.00, H 2.40, N 7.63.

Biological Materials and Methods

Cell culture and Western blot analysis

NIH 3T3 fibroblasts were purchased from ATCC. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% (v/v) bovine calf serum (ATCC) in an atmosphere of 5% CO₂ at 37°C. For testing the zinc complexes, cells were starved in DMEM (without serum) for 16 h at 37°C. Subsequent to serum deprivation, cells were treated with zinc compounds for 15 min in DMEM at 37°C. Following compound treatment, cells were stimulated either with 2 µM or 0.04 µM insulin for 15 min. A control was included where cells were neither treated with zinc compounds nor stimulated with insulin. The cells were washed afterwards three times with ice-cold PBS and then scrapped in lysis and SDS-PAGE buffer, containing 150 mM Tris-HCl, pH 6.8, 25% glycerol, 5% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue. Cell lysates were separated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% skimmed milk in TBS/T (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h at room temperature. Phospho-Akt (Ser473) rabbit monoclonal antibody, mass-Akt rabbit polyclonal antibody, GSK-3β (Ser9) rabbit polyclonal antibody and phospho-p44/42 MAPK (Thr202/Tyr204) rabbit polyclonal antibody (Cell Signaling) were used as primary antibodies for immunoblotting analysis. Primary antibody incubation was performed in 5% bovine serum albumin in TBS/T for 16 h at 4°C. Blots were washed for 15 min with

TBS/T and then incubated with horseradish peroxidase-conjugated secondary antibody (Biorad) in blocking buffer for 1h and then probed with western blotting detection reagent (ECL, GE healthcare).

PTEN expression and purification

PTEN was expressed as a glutathione S-transferase fusion-protein and purified according to methods previously described.^[26] Protein expression was induced in the *E. coli* strain XL-1 blue for 24 h using 1 mM isopropyl β -D-1-thiogalactopyranoside at 23°C. Cells were harvested and stored at -20°C. The harvested cells were re-suspended in lysis buffer containing 50 mM Tris (pH 7.4), 1% Triton X-100, 10 mM benzamidine hydrochloride, 100 μ g/mL soybean trypsin inhibitor, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride and 2 mM dithiothreitol (DTT). Lysis was performed by adding lysozyme to the cell suspension at a concentration of 2 mg/mL and sonication. Cell debris was removed by centrifugation at 18000 g for 1 h at 4°C. The supernatant was loaded onto a glutathione sepharose column, pre-equilibrated with 50 mM Tris (pH 7.4), 140 mM NaCl and 2.7 mM KCl. After loading, the column was washed twice with 50 mM Tris (pH 7.4), 140 mM NaCl, 2.7 mM KCl and 2 mM DTT. Another two washes were performed using the same buffer with 500 mM NaCl. The GST-tagged PTEN was eluted using 20 mM glutathione in 50 mM Tris (pH 7.4), 250 mM NaCl, 20% glycerol and 2 mM DTT. Protein concentration was determined using Bradford assay.

PTEN activity assay

PTEN activity in the presence of zinc compounds was assessed using the fluorescence substrate 3-O-Methylfluorescein phosphate (OMFP).^[27] OMFP was dissolved in DMSO to a concentration of 20 mM and then further diluted with 1% DMSO to the tested concentrations.

Assays were performed in 100 mM Tris (pH 7.4) containing 2 mM DTT at room temperature (20 °C). For inhibition studies PTEN was pre-incubated with zinc compounds at RT for 10 min. Reactions were then initialised by adding OMFP to a final concentration of 200 µM. The hydrolysis of OMFP to OMF was monitored by measuring the change of fluorescence units (FU) in a 96-well microtiter plate (excitation at 485 nm and emission at 525 nm) using a Varian fluorescence spectrophotometer.

Cytotoxicity assay

NIH3T3 cells were seeded into a 96-well plate at a concentration of 8000 cells per well and incubated at 37 °C for 16 hours. Cells were treated with compound **11** at concentrations between 10 nM and 1 mM for 2 h at 37°C (total volume of 100 µL). After 2h, 20 µL of a 5 mg/mL MTT solution were added to each well and incubated for further 4 h at 37°C. Media were removed carefully and 150 µL of a 4 mM HCl in isopropyl alcohol were added to each well and incubated at room temperature for 15min on an orbital shaker. The formation of formazan by viable cells was measured using a spectrophotometer at 590 nm.

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