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Synthesis and initial in vitro biological evaluation of two new zinc-chelating compounds: Comparison with TPEN and PAC-1

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ABSTRACT

The lipophilic, cell-penetrating zinc chelator *N*,*N*,*N*′,*N*′,-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, **1**) and the zinc chelating procaspase-activating compound PAC-1 (**2**) both have been reported to induce apoptosis in various cell types. The relationship between apoptosis-inducing ability and zinc affinity (K_d), have been investigated with two new model compounds, ZnA-DPA (**3**) and ZnA-Pyr (**4**), and compared to that of TPEN and PAC-1. The zinc-chelating *o*-hydroxybenzylidene moiety in PAC-1 was replaced with a 2,2′-dipicoylamine (DPA) unit (ZnA-DPA, **3**) and a 4-pyridoxyl unit (ZnA-Pyr, **4**), rendering an order of zinc affinity TPEN > ZnA-Pyr > ZnA-DPA > PAC-1. The compounds were incubated with the rat pheochromocytoma cell line PC12 and cell death was measured in combination with $ZnSO_4$, a caspase-3 inhibitor, or a ROS scavenger. The model compounds ZnA-DPA (**3**) and ZnA-Pyr (**4**) induced cell death at higher concentrations as compared to PAC-1 and TPEN, reflecting differences in lipophilicity and thereby cell-penetrating ability. Addition of $ZnSO_4$ reduced cell death induced by ZnA-Pyr (**4**) more than for ZnA-DPA (**3**). The ability to induce cell death could be reversed for all compounds using a caspase-3-inhibitor, and most so for TPEN (**1**) and ZnA-Pyr (**4**). Reactive oxygen species (ROS), as monitored using dihydro-rhodamine (DHR), were involved in cell death induced by all compounds. These results indicate that the Zn-chelators ZnA-DPA (**3**) and ZnA-Pyr (**4**) exercise their apoptosis-inducing effect by mechanisms similar to TPEN (**1**) and PAC-1 (**2**), by chelation of zinc, caspase-3 activation, and ROS production.

1. Introduction

The cellular apoptotic mechanism is a tightly regulated signaling cascade that eventually leads to the activation of caspase-3, the chief executioner caspase in the cell apoptotic pathway.[1] Zinc is the second most abundant transition metal in the human body and is involved in the catalytic function and structural stability of over 300 enzymes and proteins.[2] Manipulation of the freely available zinc has been shown to affect a great range of diseases and conditions,[3–5] and the relationship between depletion of labile zinc and activation of caspase-3 is well described; zinc is an endogenic inhibitor of procaspase-3.[6–9] The concentration of free zinc varies in biological tissues depending on zinc buffering capacity.[10] In PC12, HeLa, and HT-29 cell lines, as well as in primary cultures of cardiac myocytes and neurons in vitro, the concentration of free zinc has been determined to be approximately 5 nM.[11] The lipophilic, cell-penetrating zinc chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine[9,12,13] (TPEN, **1**, Fig. 1) has a dissociation constant (K_d) of 0.26 fM.[14] All six nitrogen atoms in TPEN coordinate to zinc in a hexadentate manner.[15] The procaspase-activating compound PAC-1 (**2**, Fig. 1), described in 2006 as the first selective procaspase-3-activating compound,[16] has also zinc-chelating properties (K_d = 42 nM).[4] The zinc-chelating property of PAC-1 is attributed to the *o*-hydroxybenzylidene-hydrazide moiety.[4,17,18] Compounds that activate caspase-3 through mechanisms other than chelation of zinc have also been reported.[19]

We hypothesized that introduction of a zinc-chelating function in PAC-1 resulting in a lower K_d than 42 nM would reflect an increased ability to induce cell death in an in vitro model cell line. With this in mind, the phenolic moiety in PAC-1 was replaced with the well described zinc-chelating unit 2,2′-dipicolylamine (DPA) in ZnA-DPA (**3**, Fig. 1), while keeping the hydrazide moiety intact. Structural variations of the DPA moiety in combination with a fourth or fifth donor atom, has been reported to afford lipophilic and selective zinc chelators,[20–22] displaying K_d values in the range 1–10 nM. Next, in ZnA-Pyr (**4**, Fig. 1), the phenolic moiety in PAC-1 was replaced with a 4-pyridoxyl group.[23–26] A 4-pyridoxyl moiety also comprising the *o*-hydroxybenzylidene-hydrazide moiety has an estimated K_d with zinc of approximately 200–300 fM,[23] an order

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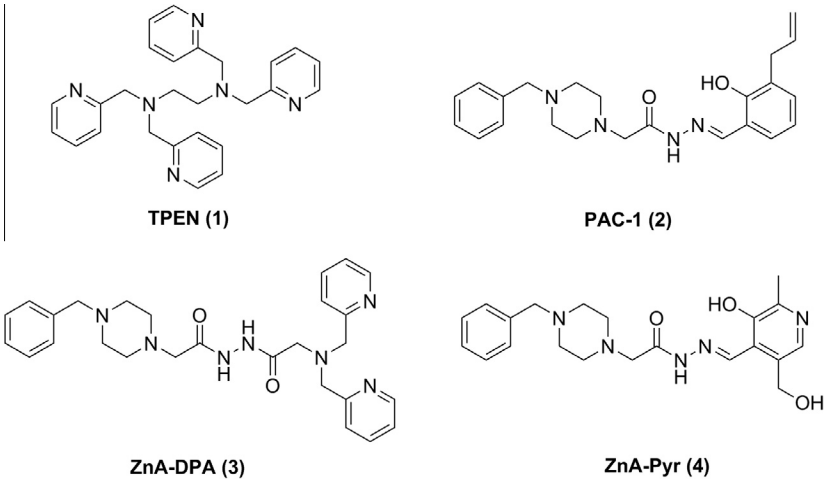


Figure 1. Overview of the compounds in this study.

of magnitude lower than for ZnA-DPA. Consequently, the anticipated order of zinc affinity for the four test substances is TPEN > ZnA-Pyr > ZnA-DPA > PAC-1.

As lipophilicity affects cell membrane penetration, we calculated the log*P*-values for the model compounds and compared them to the literature values for TPEN and PAC-1. TPEN is an inducer of apoptosis involving ROS.[12] However, it is to our knowledge not reported whether the apoptotic effects of PAC-1 involves ROS. Thus, the aims of the present study were twofold: 1) to investigate the ability of ZnA-DPA (**3**) and ZnA-Pyr (**4**) to induce cell death and caspase-3 activation compared to TPEN (**1**) and PAC-1 when the zinc-chelating *o*-hydroxybenzylidene-hydrazide moiety in PAC-1 was exchanged with two metal-chelating functionalities with lower K_d. 2) to investigate if cell death induced by TPEN (**1**), PAC-1, ZnA-DPA (**3**) or ZnA-Pyr (**4**), correlated to other parameters such as zinc concentration, caspase-3 activation, or ROS production.

2. Results and discussion

2.1. Chemistry

The new zinc chelators were synthesized according to Scheme 1. All new compounds exhibited spectra in accordance with their anticipated structures. Purity was analysed using a HPLC system and all final products were $\geqslant$95% pure (not shown).

2.2. Zinc binding

The zinc-binding ability of ZnA-Pyr (**4**) was assessed using UV spectroscopy. ZnA-DPA (**3**) contained no UV-sensitive chromophore that gave a significant response when treated with zinc and could therefore not be determined spectrophotometrically. ZnA-Pyr (**4**), however, showed a dose-response in absorbance when treated with zinc (Fig. 2). Literature values[20,21] for zinc affinity for analogous compounds were used qualitatively for discussion purposes in the present study.

2.3. Biological evaluation

Two new compounds, ZnA-DPA (**3**) and ZnA-Pyr (**4**), were synthesized with the aim of comparing them to the reported procaspase-3 activators PAC-1 and TPEN (**1**). The design of the new compounds included stronger zinc binding motifs as compared to PAC-1, while keeping the rest of the molecule intact. However, these molecular modifications did lead to a significant reduction in log$D_{7.4}$ by a factor of roughly two for ZnA-DPA (**3**) (log$D_{7.4}$ = 1.40) and ZnA-Pyr (log$D_{7.4}$ = 1.27) as compared to PAC-1 (log$P$ = 3.43).[27] The increased zinc-binding affinity of ZnA-DPA (**3**) and ZnA-Pyr compared to PAC-1, as expressed by reduced K_d, will therefore be offset by the reduction in lipophilicity, since smaller portion of the chelators will be able to cross the cell membranes and affect the cell zinc homoeostasis. This is evident by the observed reduction in toxicity. Despite the differences in physical properties, we sought to investigate the cell toxicity and the mechanism of toxicity for all compounds.

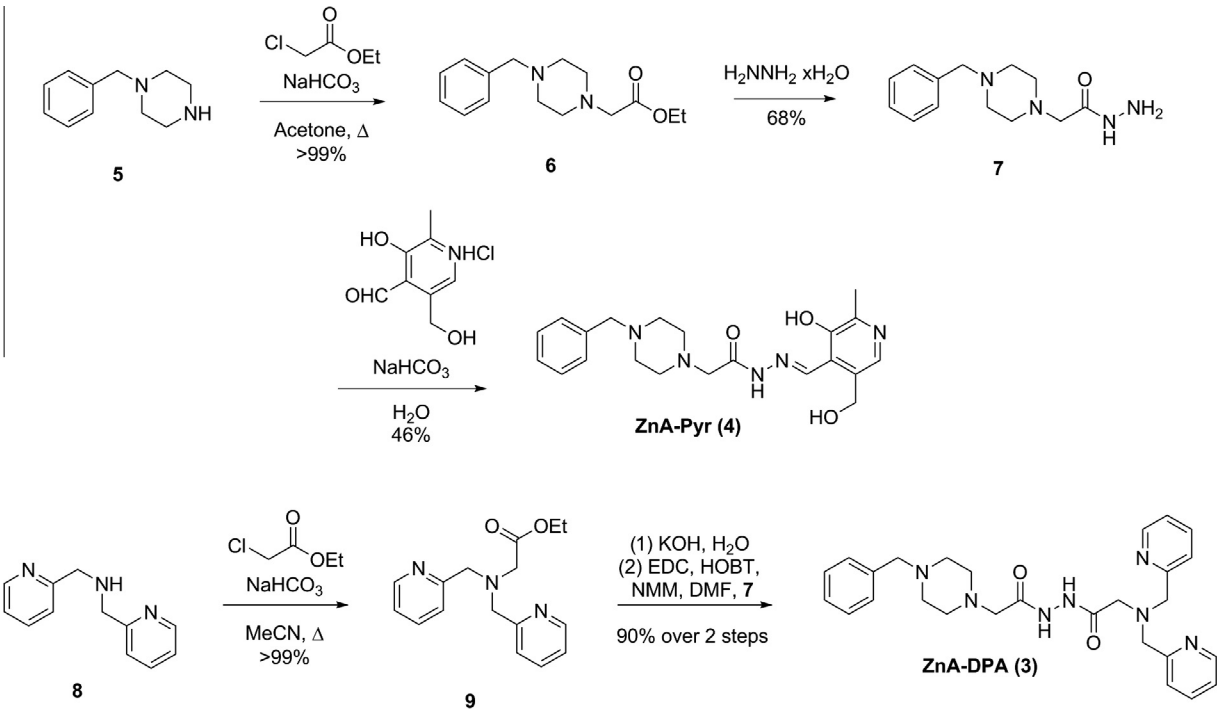
2.3.1. Concentration dependant cell death

TPEN (**1**) and PAC-1 (**2**) induced significant cell death in PC12 cells; as compared to control in a concentration range 25–200 μM, in a concentration dependent manner (Fig. 3). ZnA-DPA (**3**) and ZnA-Pyr (**4**) also induced cell death but significant induction was only observed at higher concentration, 75–200 μM (Fig. 3). The negative control, treated only with DMSO, showed no induction of cell death at any of the concentrations used (not shown). The EC_{50} values for TPEN (**1**) and PAC-1 (**2**) were calculated to be 38 and 122 μM, respectively, using Graph Pad Prism software. EC_{50} values for ZnA-DPA (**3**) and ZnA-Pyr (**4**) could not be calculated because of solubility problems above 200 μM.

2.3.2. Procaspase-3 activation

Since PAC-1 is reported to induce toxicity by disrupting the zinc homeostasis and activation of caspase-3,[4] we sought to investigate the role of caspase-3 activation by all tested compounds. The PC12 cells were cultured in 96 well black plates at cell density 28×10^4 for 24 h and then exposed to TPEN (**1**) (25 μM), PAC-1 (**2**) (100 μM), ZnA-DPA (**3**), or ZnA-Pyr (**4**) (both 200 μM) for four hours, concentrations where they exhibited a comparable level of cell death. Cells treated with DMSO were used as control. Staurosporine 0.5 μM and 1 μM concentrations were used as a positive control to activate caspase-3. Results presented in Fig. 4 show that all the compounds activated caspase-3 over the control level, but less than staurosporine, which gave 6000 and 30,000 RFU/million cells, using 0.5 or 1 μM, respectively (not shown).

ZnA-Pyr (**4**) and ZnA-DPA (**3**) do produce significant amounts of caspase-3 relative to control, albeit less than TPEN (**1**) and PAC-1 (**2**). It is also here reasonable to assume that lipophilicity affects these results.



Scheme 1. Synthesis of PAC-1 analogs ZnA-Pyr (**4**) and ZnA-DPA (**3**).

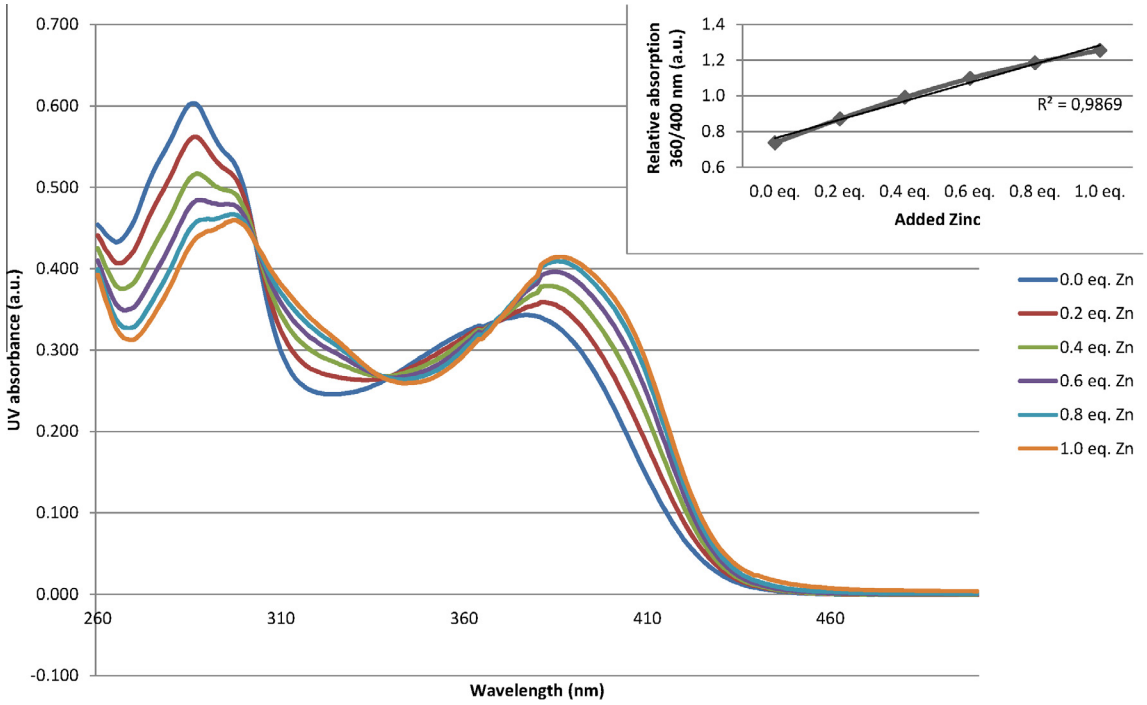


Figure 2. Zinc chelation with ZnA-Pyr (**3**). Dose dependent response of ZnA-Pyr (50 μM) when treated with 0–50 μM $ZnSO_4$ in Hepes buffer (pH 7.4) is shown. Inset: The relative increase in absorption at 360 nm and 400 nm in response to added $ZnSO_4$.

2.3.3. Reactive oxygen species

ROS production could also be a major cause of toxicity, either up-stream or down-stream of caspase-3 activation, and so the ability to produce ROS was detected using dihydrorhodamine (DHR) and dihydroethidium (DHE) as ROS probes. Relative ROS production using DHR as probe (Fig. 5) was higher as measured one hour after addition of the compounds compared to four hours, and TPEN (**1**), PAC-1 (**2**), and ZnA-Pyr (**4**), but not ZnA-DPA (**3**), showed significantly higher DHR oxidation than vehicle control at this time-point. None of the compounds showed significant difference to the DMSO control after 4 h, indicating a rapid and transient ROS induction. DHE, however, did not detect any ROS production with any of the compounds (results not shown).

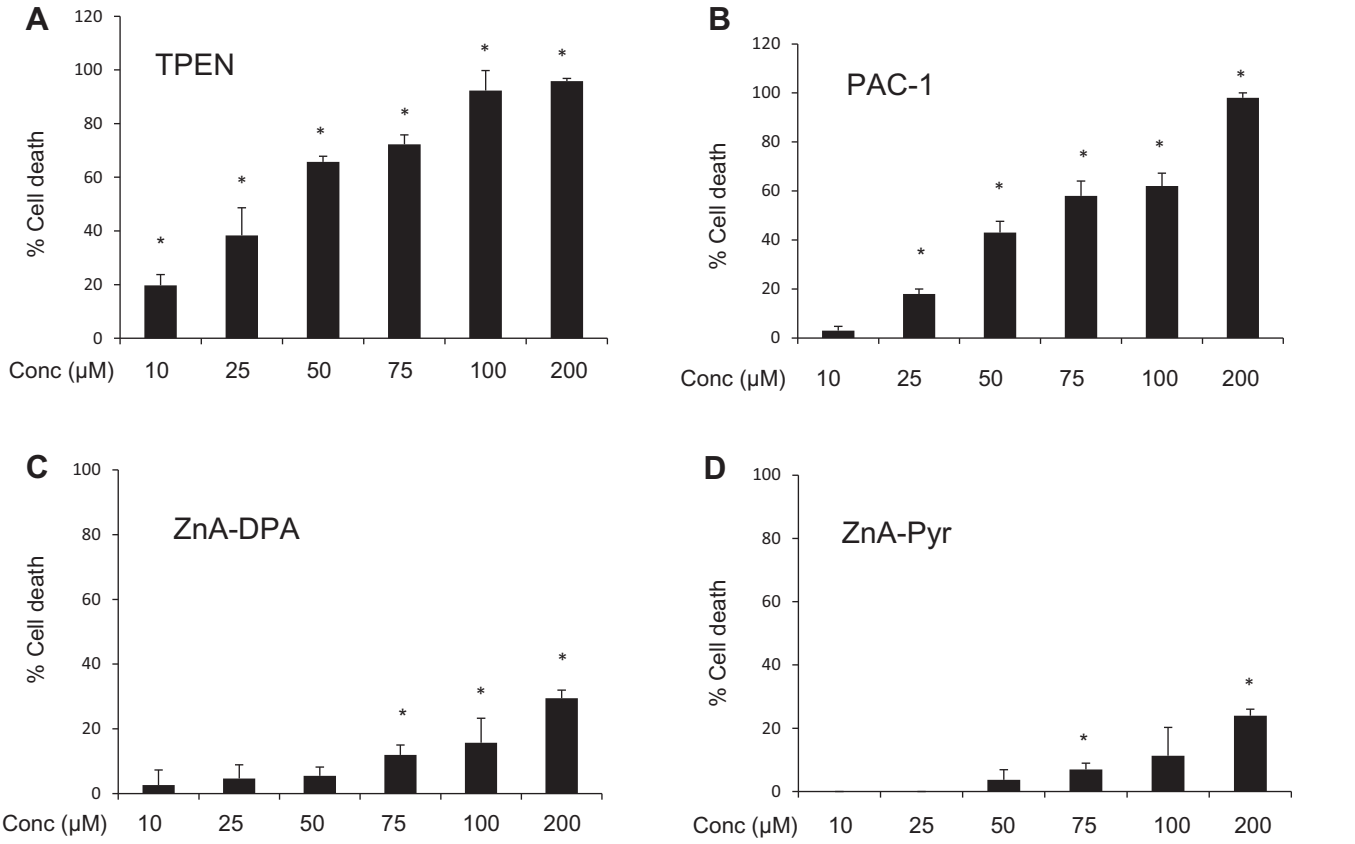


Figure 3. Induction of cell death in PC12 cells. PC12 cells were treated with TPEN, PAC-1, ZnA-DPA, or ZnA-Pyr. Cell death is expressed as percentage relative to the total number of cells. The results are average of 4 experiments ± S.D. Significant difference is shown as compared to DMSO control (*), $P < 0.05$, one-way ANOVA with Holm-Sidak post hoc method.

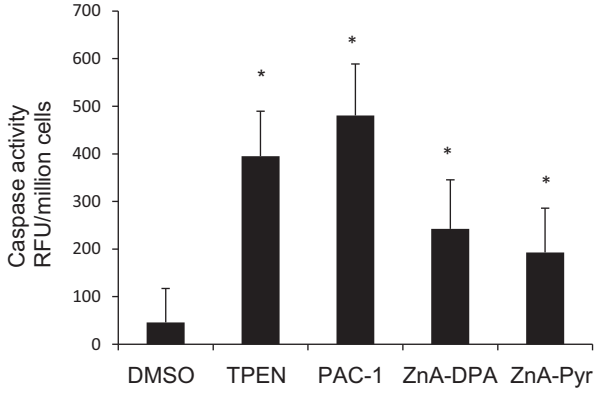


Figure 4. Caspase-3 activity. PC12 cells were treated with TPEN (25 μM), PAC-1 (100 μM), ZnA-DPA, or ZnA-Pyr (200 μM). Cells treated with DMSO were used as control. Results are expressed as average of 5 experiments ± S.D. Significant difference as compared to control is shown by *. Significance is $P < 0.05$, one-way ANOVA with Holm-Sidak method.

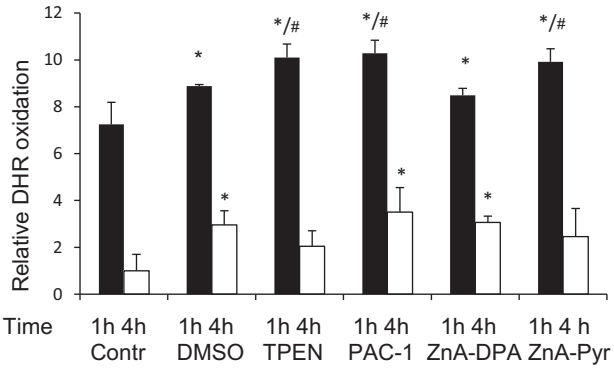


Figure 5. Induction of ROS in PC12 cells. PC12 cells were loaded with DHR (0.1 μM) for 30 min, and left untreated or treated with DMSO (vehicle control), TPEN (25 μM), PAC-1 (100 μM), ZnA-DPA, or ZnA-Pyr (200 μM) for one hour or four hours followed by reading the slope for ROS production for 30 min. Results are expressed relative to the untreated control slope at 4 h, as average of 3 experiments ± S.D. Significant difference as compared to untreated is shown by * and to DMSO by #, $P < 0.05$, one-way ANOVA with Holm-Sidak post hoc testing.

2.3.4. Reduction in cell death by inhibitors

Since caspase-3 activation and ROS production were detected with all compounds, it was of interest to determine how addition of inhibitors would affect the toxicity. Therefore, DHR and caspase-3 inhibitor were used as inhibitors of cell death. $ZnSO_4$ was used to determine whether an increase in the zinc level could reduce cell death. Cells were exposed to TPEN (**1**) (25 μM), PAC-1 (**2**) (100 μM), ZnA-DPA (**3**), or ZnA-Pyr (**4**) (both 200 μM). Cells were treated with compounds alone and in combination with $ZnSO_4$, caspase-3 inhibitor, or DHR used as ROS scavenger. Cells were exposed for 24 h and then stained with trypan blue (Fig. 6). No cell death was observed with the inhibitors alone. TPEN (**1**) caused cell death that was significantly reduced by $ZnSO_4$ or caspase-3 inhibitor. For PAC-1 (**2**) and ZnA-Pyr (**4**) the addition of DHR, $ZnSO_4$ and caspase-3 inhibitor gave a significant reduction of cell death, whereas ZnA-DPA (**3**) caused cell death that was significantly prevented by DHR and caspase-3 inhibitor, but less significant by $ZnSO_4$ compared to the other compounds. Different

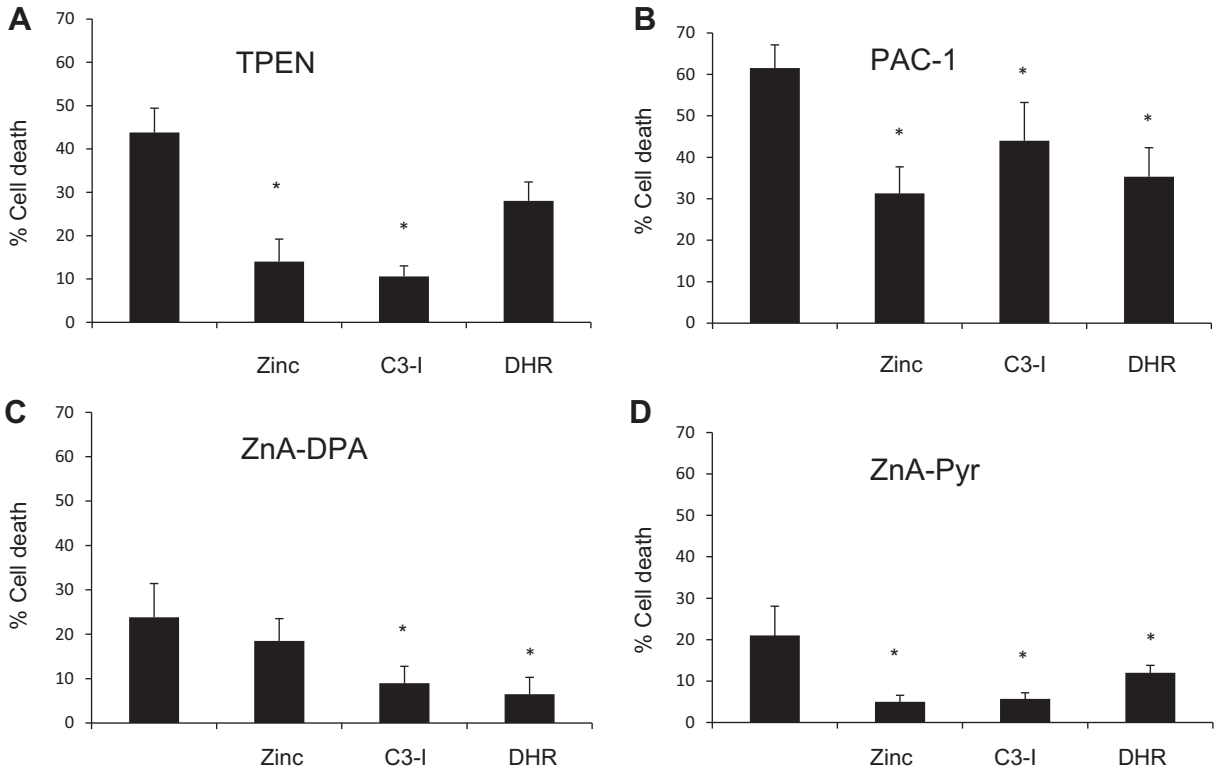


Figure 6. Inhibition of cell death with zinc, caspase-3 inhibitor, or DHR. PC12 cells were exposed to TPEN (25 μM), PAC-1 (100 μM), ZnA-DPA, or ZnA-Pyr (200 μM), alone and with inhibitors $ZnSO_4$ (zinc, 10 μM), caspase-3 inhibitor (C3-I, 1 μM), or DHR (1 μM). Cell death was counted as percentage relative to the total number of cells and results are expressed as average from 4 experiments + S.D. Results were significant at $P < 0.05$ (One-way ANOVA with Holm-Sidak post hoc method). Significance was calculated based on difference compared to DMSO and are showed by (*).

levels of reduction in toxicity were seen with caspase-3 inhibitor. The reduction for 100 μM PAC-1 (**2**) was roughly 33%, but for 200 μM ZnA-Pyr (**4**) it was 66%.

As can be seen when adding DHR to the experiments, ROS is an important contributor to toxicity for these compounds. The toxicity of PAC-1 (**2**), ZnA-DPA (**3**) and ZnA-Pyr (**4**) was significantly reduced with roughly the same factor.

2.3.5. Mechanism of toxicity

Disruption of the zinc homeostasis seems to be the primary mechanism of toxicity for all four model compounds. The reduction in cell death observed by addition of zinc was similar or greater than by addition of a caspase-3 inhibitor or a ROS scavenger. Regarding the new compounds, ZnA-Pyr (**4**) is a stronger zinc binder than ZnA-DPA (**3**) and the cell toxicity of 200 μM ZnA-Pyr (**4**) is significantly more reduced upon addition of 10 μM $ZnSO_4$ than for 200 μM ZnA-DPA (**3**). The relative reduction in toxicity upon addition of zinc to ZnA-Pyr (**4**) is even larger than for PAC-1 (**2**), but not TPEN (**1**), which has the highest affinity for zinc, and is administered as 25 μM. Thus, the added amount of zinc relative to the test concentrations of the model compounds indicates that the absolute reduction in toxicity correlates with the K_d values for the compounds.

3. Conclusion

The new compounds, ZnA-DPA (**3**) and ZnA-Pyr (**4**), induce procaspase-3 activation, which in turn leads to cell death. They are less lipophilic than the parent compounds TPEN (**1**) and PAC-1 (**2**), and would therefore presumably not cause significant neurotoxicity at relevant doses, as recently found for PAC-1.[28] They are, however, only mildly toxic to PC12 cells. Toxicity seems to correlate well with lipophilicity, indicating limited cell penetration for ZnA-DPA (**3**) and ZnA-Pyr (**4**). When comparing ZnA-DPA (**3**) and ZnA-Pyr (**4**), the latter was a better zinc chelator and more sensitive to the zinc level in the cells. However, disturbance of zinc homeostasis seems to be the primary mechanism of toxicity for all four model compounds, since cell death can be reduced not only with zinc or caspase inhibitors but also with a ROS scavenger.

4. Experimental procedures

4.1. Materials

Caspase-3 fluorometric substrate ($(DEVD)_2$-Rhodamine110) was purchased from Bachem AG (Switzerland), and caspase-3 inhibitor (Ac-DEVD-cmk) was purchased from Calbiochem (San Diego, CA). TPEN was purchased from TCI Europe and was used without further analysis. Dulbecco's modified Eagle's medium (DMEM), horse serum, and fetal bovine serum were purchased from Gibco (Paislet, Scotland). All other reagents were from Sigma (St. Louis, USA).

4.2. Methods

4.2.1. Zinc titration

Compounds were diluted from their 10 mM DMSO stock solutions into 3 mL of buffer (50 mM Hepes, 100 mM $NaNO_3$, pH 7.4 to give a final concentration of 50 μM in quartz cuvettes. Then 10–50 μL of a 3 mM $ZnSO_4$ solution in buffer was added to the cuvettes in order to make samples with 0–1 equiv of added ZnSO4 (50 mM Hepes, 100 mM $NaNO_3$, pH 7.4). The solutions were mixed and allowed to equilibrate for 60 min. The absorbance spectra

between 260 and 500 nm were then acquired using a spectrophotometer (Biochrom Libra S32PC).

4.2.2. Biological evaluation and toxicity measurement

PC12 cells (a rat pheochromocytoma cell line) were grown in DMEM 7emented with 5% (v/v) horse serum, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin mixture (Gibco) and 1% (v/v) sodium pyruvate (Gibco). Cells were seeded in 58 mm dishes (3.3 mL/dish at a density of 350,000 cells/mL). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

4.2.3. Cell death assays

PC12 cells were treated with TPEN (**1**), PAC-1 (**2**), ZnA-DPA (**3**), or ZnA-Pyr (**4**) at 10, 25, 50 75, 100, and 200 μM concentrations, dissolved in DMSO (0.1% final concentration). Cell death was measured with trypan blue exclusion after 24 h of treatment. Trypan blue in 0.9% NaCl solution was added 1:4 into the medium and the cells were incubated for 30 min at 37 °C. Cells excluding the dye (unstained cells) were scored as viable and the cells stained with dye (blue cells) were scored as dead. Untreated and DMSO treated cells were used as control.

4.2.4. Caspase-3 activity assay

For detection of caspase-3 activity cells were treated with TPEN (**1**) (10 μM), PAC-1 (**2**) (100 μM), ZnA-DPA (**3**) (200 μM), ZnA-Pyr (**4**) (200 μM), or DMSO directly in black 96 well plates for four hours. Modified RIPA buffer (50 mM Tris–HCl (pH 7.4), 1% Igepal, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EGTA) supplemented with 20 μM of caspase-3 fluorometric substrate $(DEVD)_2$-Rhodamine 110 was added in four wells. Another two wells were added RIPA buffer supplemented with substrate and 1 μM caspase-3 inhibitor Ac-DEVD-cmk (negative control). There was more than 85% reduction in caspase-3 activity with 1 μM caspase-3 inhibitor added to the cells or added in the assay. The cell lysate was incubated for 24 h at 37 °C before fluorescence was measured at excitation 485 nm and emission 535 nm, using a fluorometer (Perkin–Elmer HTS 7000 Plus. Bio Assay Reader). There was a gradual increase in fluorescence with time in this period. This method was adopted from literature.[28,29]

4.2.5. Reactive oxygen species detection assay

To determine the effects of TPEN (**1**), PAC-1 (**2**), ZnA-DPA (**3**), and ZnA-Pyr (**4**) on reactive oxygen species generation, cells were cultured in black 96 well plates. Cells were incubated with DHR (0.1 μM) for 30 min at 37 °C, and washed with medium before treatment with TPEN (**1**) (10 μM), PAC-1 (**2**) (100 μM), ZnA-DPA (**3**) (200 μM), or ZnA-Pyr (**4**) (200 μM) for one hour or four hours. Medium was replaced with PBS and the plate was measured for 30 min at excitation 485 nm and emission 535 nm, using a fluorometer (Perkin–Elmer HTS 7000 Plus. Bio Assay Reader). The slope was used in the calculation of ROS production.

4.2.6. Statistical analysis

Statistical differences were analysed by one-way repeated measures analysis of variance (ANOVA) followed by Holm-Sidak post hoc test (Sigma Stat software international, Ashburn, VA). A P value of <0.05 was considered significant.

4.3. Synthesis

PAC-1 was synthesized in our laboratory according to literature.[16] The prepared compounds were identified by ^1H, ^{13}C NMR spectroscopy, and HRMS, and shown to be >95% pure by HPLC analysis. High resolution mass spectrometry was obtained using an Autospec Ultima GC-MS (Micromass Ltd. Manchester, England). The MS was equipped with an electron ionization (EI) ion source producing 70 eV electrons. The voltage scan time was 1 s. and the inter scan delay time was 0.20 s. The mass spectrometer was tuned to a resolution of 12,000. The ion source temperature was set to 200 °C and the samples were introduced into the instrument via a direct insertion probe, which was cooled with water. The software used was MassLynx version 4.0. (Waters, Milford, MA, USA).

4.3.1. Synthesis of ethyl 2-(4-benzylpiperazin-1-yl)acetate (6)

Ethyl chloroacetate (1.14 mL, 11.0 mmol, 1.1 equiv) in 10 mL acetone was added to a stirring solution of 1-benzylpiperazine (**5**) (1.73 mL, 10.0 mmol, 1.0 equiv) in 10 mL acetone. Sodium hydrogen carbonate (1.05 g, 12.5 mmol, 1.25 equiv) was then added to the reaction mixture with the aid of 5 mL acetone. The suspension was then heated to reflux and left for 22 h at which point no starting material was visible on TLC (2:1 hexane/EtOAc). The mixture was filtered and the salt was washed with 3 × 5 mL acetone. The pale yellow filtrate was then concentrated under reduced pressure to give 2.67 g of a yellow oil. The crude product was purified by column chromatography (130 g of SiO_2 using 1:1–2:1 EtOAc/hexane) affording 2.29 g (87%) of a pale yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.39–7.17 (m, 5H), 4.18 (q, J = 7.1 Hz, 2H), 3.52 (s, 2H), 3.20 (s, 2H), 2.81–2.36 (m, 8H), 1.26 (t, J = 7.1 Hz, 3H). The spectroscopic data obtained are in accordance with published data.[16]

4.3.2. Synthesis of 2-(4-benzylpiperazin-1-yl)acetohydrazide (7)

Hydrazine hydrate (2.22 mL 80%, 36.63 mmol, 3.0 equiv) was added drop wise to a stirring solution of ethyl 2-(4-benzylpiperazin-1-yl)acetate (**6**) (3.20 g, 12.21 mmol, 1.0 equiv) in 17 mL absolute ethanol. After the addition, the mixture was heated to reflux and left overnight. The pale yellow solution was concentrated under reduced pressure and mixed with 20 mL (1:1 brine/water pH >12) and extracted with 3 × 15 mL CH_2Cl_2 followed by 15 mL EtOAc. The combined organic phases were pooled and dried over $MgSO_4$, filtered and concentrated under reduced pressure to give 3.24 g of a thick pale oil. It was dissolved in EtOH and left overnight in a 1:15 EtOH/Et_2O solution to precipitate needle like white crystals. The mother liquor was then concentrated, dissolved in EtOH and poured into Et_2O to give a total yield of 2.96 g (97%). ^1H NMR (300 MHz, $CDCl_3$) δ 8.12 (s, 1H), 7.40–7.18 (m, 6H), 3.84 (d, J = 4.6 Hz, 2H), 3.51 (s, 2H), 3.07 (s, 2H), 2.64–2.31 (m, 11H). The spectroscopic data obtained are in accordance with published data.[16]

4.3.3. Synthesis of (*E*)-2-(4-benzylpiperazin-1-yl)-*N*′-((3-hydroxy-5-(hydroxymethyl)-2-methylpyridin-4-yl)methylene)acetohydrazide (4, ZnA-Pyr)

The hydrazide 2-(4-benzylpiperazin-1-yl)acetohydrazide (**7**) (25 mg, 1.0 mmol, 1.0 equiv), pyridoxal hydrochloride (22 mg, 0.11 mmol, 1.1 equiv) and sodium carbonate (7 mg, 0.13 mmol, 1.3 equiv) were mixed and dissolved in 2 mL water whereupon the mixture turned yellow. The mixture was heated to 80 °C and left for 90 min after which a white solid started precipitating. The mixture was cooled to room temperature and extracted with 3 × 2 mL CH_2Cl_2 (in which the solid dissolved). The combined organic phases was washed with 3 mL H_2O, dried over $MgSO_4$ and concentrated to a yellow solid affording 12 mg (40%) of the title compound. ^1H NMR (400 MHz, $CDCl_3$) δ 11.51 (s, 1H), 10.25 (s, 1H), 8.69 (s, 1H), 7.76 (s, 1H), 7.40–7.17 (m, 5H), 4.70 (s, 2H), 3.54 (s, 2H), 3.16 (s, 2H), 2.55 (m, 11H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.5, 151.9, 150.3, 146.3, 138.8, 137.9, 131.3, 129.3, 128.5, 127.4, 120.2, 63.0, 60.95 (d, J = 8.4 Hz), 53.9, 53.0, 19.2. HRMS (EI) calcd for $C_{21}H_{27}N_5O_3$ 397.2114, found 397.2099.

4.3.4. Synthesis of ethyl 2-(bis(pyridin-2-ylmethyl)amino)-acetate (9)

2,2′-Dipicolylamine (**8**) (199 mg, 1.0 mmol, 1.0 equiv) was diluted in 1 mL acetonitrile and mixed with sodium carbonate (75 mg, 1.2 mmol, 1.2 equiv). Ethyl chloroacetate (135 mg, 1.1 mmol, 1.1 equiv) in 1 mL acetonitrile was then added drop wise to the stirring solution. The reaction was then heated to reflux for 18 h. The reaction mixture was filtered to remove inorganic impurities and the filter cake washed with 3 × 5 mL diethyl ether. The combined organic phases were concentrated to give 338 mg of dark orange oil. The crude product was then purified by column chromatography (SiO_2 column with 10% MeOH in CH_2Cl_2 as eluent) to afford 263 mg (quantitative) of a rust red oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58–8.46 (m, 2H), 7.69–7.60 (m, 2H), 7.56 (d, J = 7.7 Hz, 2H), 7.18–7.09 (m, 2H), 4.21–4.09 (m, 2H), 4.00 (d, J = 6.3 Hz, 4H), 3.48 (dd, J = 6.3, 3.9 Hz, 2H), 1.32–1.18 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4, 159.3, 149.2, 136.6, 123.3, 122.2, 60.6, 60.1, 55.2, 14.4. The spectroscopic data obtained are in accordance with published data.[30]

4.3.5. Synthesis of 2-(4-benzylpiperazin-1-yl)-*N*′-(2-(bis(pyridin-2-ylmethyl)amino)acetyl)acetohydrazide (3, ZnA-DPA)

The ester ethyl bis(pyridin-2-ylmethyl)glycinate (**9**) (522 mg, 1.83 mmol, 1.0 equiv) was dissolved in 2 mL H_2O and 2 mL 5 M KOH solution. The mixture was stirred at room temperature for 60 min and then the pH was adjusted to 7 with 1 M HCl. The solution was concentrated under reduced pressure to a pale orange sticky solid and it was washed with 3 × 10 mL hot absolute ethanol. The combined ethanol was then concentrated under reduced pressure to give a dark red oil which was used without purification in the next step. 2-(4-Benzylpiperazin-1-yl)acetohydrazide (**3**) (64 mg, 0.26 mmol, 1.0 equiv) was mixed with the crude acid from the previous step (80 mg, 0.27 mmol, 1.03 equiv), *N*-methyl morpholine (65 μL, 0.57 mmol, 2.2 equiv), HOBT (8 mg, 0.05 mmol, 0.2 equiv), diluted in 1 mL absolute ethanol and cooled to 10 °C. EDC (60 mg, 0.31 mmol, 1.2 equiv) was then added to the stirring solution. The solution was then heated to room temperature and stirred for four hours. The mixture was then concentrated under reduced pressure, diluted in 5 mL H_2O and extracted with 3 × 5 mL CH_2Cl_2. The combined organic phases were dried over $MgSO_4$ and concentrated to give a pale orange oil. The crude product was purified by column chromatography (neutral Al_2O_3, eluent 5–10% MeOH in EtOAc) to afford 179 mg (90%) as a pale orange oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.04 (s, 1H), 9.30 (s, 1H), 8.57 (d, J = 4.7 Hz, 2H), 7.58 (td, J = 7.7, 1.7 Hz, 2H), 7.34–7.18 (m, 7H), 7.13 (dd, J = 6.9, 5.3 Hz, 2H), 3.92 (s, 4H), 3.51 (d, J = 8.4 Hz, 4H), 3.15 (s, 2H), 2.73–2.38 (m, 8H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.7, 166.7, 158.4, 149.4, 138.1, 136.8, 129.3, 128.3, 127.2, 123.2, 122.5, 63.0, 60.9, 60.3, 57.7, 53.8, 53.2. HRMS (EI) calcd for $C_{27}H_{33}N_7O_2$ 487.2696, found 487.2722.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.06.037. These data include MOL files and InChiKeys of the most important compounds described in this article.

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