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Naturally Inspired Peptide Leads: Alanine Scanning Reveals an Actin-Targeting Thiazole Analogue of Bisebromoamide

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Abstract: Systematic alanine-scanning of the linear peptide bisebromoamide (BBA), isolated from a marine cyanobacterium, is enabled by targeting the solid phase peptide synthesis of thiazole analogues. The synthetic Tz-BBA analogues have comparable cytotoxicity (nM) to bisebromoamide and cellular morphology assays indicate that they target the actin cytoskeleton. Pathway inhibition in human colon tumour (HCT-116) cells has been explored using reverse phase protein array (RPPA) analysis, which shows a dose-dependent response of IRS-1 expression. Alanine-scanning reveals a structural dependence to the cytotoxicity, actin-targeting and pathway inhibition, and allows a new readily-synthesised lead to be proposed.

Introduction

Non ribosomal peptide synthase (NRPS)-derived natural products provide a wealth of therapeutic leads due to the chemical and structural diversity which they encompass.[1] Since NRPS peptides often contain heavily modified analogues of the proteinogenic amino acids which make up their ribosomal counterparts,[2] they tend to be more resistant to proteolysis and thus ripe for development as drugs. One of the greatest challenges to studying the bioactivity of NRPS peptide leads is their synthesis, and the modification of this synthetic process to allow systematic structure-activity relationship (SAR) studies to be conducted.[3,4] Reducing this challenge to two simpler problems of: (i) efficient building block synthesis; and (ii) building block coupling under standard solid phase peptide synthesis (SPPS) conditions, provides an attractive approach and facilitates SAR studies (e.g. peptide scanning)[5] which are commonly employed in the proteinogenic domain.

The linear peptide bisebromoamide [BBA (1), Scheme 1 (a)] was isolated from a marine cyanobacterium Lyngbya sp. by the Suenaga group in 2009.[6] BBA was shown to have an IC_{50} of 0.04 µg/mL against HeLa S_2 cells and an average GI_{50} of 40 nM when tested against a panel of 39 human cancer cell lines.[7] BBA has been shown to inhibit the phosphorylation of ERK in NRK cells,[8] and both ERK and Akt in two renal cell carcinoma lines.[9] BBA has also been shown to be the first linear peptide to act as an actin filament stabilizer.[10] While the biosynthesis of BBA 1 has not yet been elucidated, the presence of D-amino acids and heavily modified proteinogenic amino acids led Suenaga et al. to suggest a NRPS origin.[2,8] To date, two total syntheses have been reported[10] both of which rely on stepwise construction of tri-peptide fragments in solution, followed by late stage introduction, or construction, of the sensitive thiazoline (Tzl) ring [Scheme 1 (b)]. These prior studies have generated a very limited amount of SAR data, and indicate that either enantiomeric orientation of the methyl group on the thiazoline ring is equally active.[10] Crucially, subsequent isolation of the nor-methyl analogue at this position (nor-BBA), has shown that it also retains activity.[9] These data allowed us to design a more pH stable thiazole analogue[11] of bisebromoamide [Tz-BBA (Bis1), Scheme 1 (c)] with confidence, and to propose an SPPS route to its construction which would facilitate systematic investigation of the SAR of this intriguing peptide.

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Supporting information for this article is available via a link at the end of the document. Primary data files can be found at http://dx.doi.org/10.7488/ds/1417
Results and Discussion

Amide-bond disconnection across Tz-BBA Bis1 led to the identification of seven key fragments: aa\(^1\) 2-(1-oxo-propyl)pyrrolidine (Opp); aa\(^2\) N-methyl phenylalanine (NMephe); aa\(^3\) d-leucine (d-Leu); aa\(^4\),\(^5\) the cyclised and oxidised conjugate of 4-methylproline and cysteine (4-MePro-Tz); aa\(^6\) N-methyl-3-bromotyrosine (NMMe-d-BrTyr); aa\(^7\) alanine (Ala); and pivalic acid (Piv) which caps the N-terminus. Targeting an SPPS based strategy for synthesis required each of the six amino acid-based fragments in nitrogen protected forms. Practical issues associated with the amino acid sequence\(^{[12]}\) and linker group compatibility compelled the usage of Boc-protecting groups. The three non-commercial fragments were synthesised as shown in Scheme 2. Grignard addition to commercial Weinreb amide 2\(^{[13]}\) gave the Opp fragment 3 in excellent yield (97%). Synthetic 4-methyl-proline tert-butyl ester 4\(^{[14]}\) was converted to Boc carbamate 5 under standard conditions, and DIBAL-H reduction of the ester to give prolinol derivative 6 proceeded smoothly. The resultant aldehyde was coupled to L-cysteine methyl ester following the precedent of Shiori et al.\(^{[19]}\) to give intermediate thiazolidine 7 as a mixture of diastereomers (1:1.5 by NMR). Reproducible oxidation to the corresponding thiazole 8 (MnO\(_2\)) was shown to be dependent on pre-activation of the oxidant by heating.\(^{[15]}\) Basic hydrolysis of the methyl ester gave the 4-MePro-Tz fragment 9 (55% overall yield from 4) in excellent optical purity. Finally, the phenol in bromo-tyroline derivative 10 (readily accessed using selective mono ortho-bromination by NBS)\(^{[16]}\) was protected as its tert-butyl ether 11 using a modification of the procedure reported by Sambri et al.\(^{[17]}\) N-methylation to give 12 and subsequent ester cleavage gave the desired NMMe-d-BrTyr fragment 13 (66% overall yield from 10).

The use of a hydrazine-based linker\(^{[18]}\) was required to facilitate the direct attachment of the C-terminal Opp moiety in the SPPS synthesis of Tz-BBA Bis1. The hydrazide adducts which result are stable to basic and non-aqueous acidic conditions, show little evidence of racemisation and provide products in high levels of purity. We were attracted to the semicarbazide linker described by Albericio et al.\(^{[19]}\) which has been used by several groups for the synthesis of peptidyl aldehydes and ketones.\(^{[20]}\) Modified resin 14 was readily prepared from aminomethyl-poly(styrene) by treatment with CDI followed by tert-butyl carbazate (Scheme 3);\(^{[19]}\) successful TFA-mediated deprotection of intermediate species 15 was confirmed using the TNBS test.\(^{[19]}\) The Boc-protected Opp moiety 3 was attached as its hydrazide in the presence of acetic acid.\(^{[20a]}\) Subsequent amino acid coupling cycles were achieved using anhydrous conditions for Boc deprotection, followed by Oxyma/DIC-mediated coupling with the appropriate amino acid.\(^{[20b]}\) In all cases, only three equivalents of the coupling amino acid were used, but where the N-terminus was comprised of a secondary amino acid a double coupling cycle was employed. For the final coupling and capping steps [after the addition of Boc-BrTyr(O\(_{Bu}\))], a two-step deprotection process was employed to allow selective cleavage of the Boc carbamate in the presence of the tert-butyl ether.\(^{[21]}\) Cleavage from the resin and deprotection were achieved under aqueous acidic conditions\(^{[19]}\) allowing the desired product Tz-BBA Bis1 to be isolated, purified by RP-HPLC\(^{[22]}\) and analyzed by 2D NMR.\(^{[1,2]}\) NMR data for the 4-MePro ring shows marked differences between cis- and trans-substituted isomers;\(^{[14]}\) detailed analysis of NMR data for peptides Bis1-6 supported the conclusion that negligible epimerization of the 4-MePro-Tz motif had occurred during SPPS.
Peptide scanning, like SPPS, is a technique used more commonly in the development of ribosomal peptides, however it can also be used for non-ribosomal species and it provides a wealth of information.[9] The limited studies on BBA (1) to date have not allowed a comprehensive assessment of the activity which results from each individual amino acid residue,[8,10] most probably because of the relative complexity of previous synthetic routes, combined with the competitive instability of amino acids adjacent to a thiazoline moiety. By systematically replacing amino acid residues \( \text{aa}^2, \text{aa}^3 \), and \( \text{aa}^4 \) in the Tz-BBA target \( \text{Bis}1 \) with alanine,[24] three analogues \( \text{Bis}3-5 \), Table 1) were successfully synthesized by SPPS using the semicarbazide resin (2-8% overall yield). Replacement of the C-terminal Opp moiety with alanine \( \text{Bis}2 \), Table 1) was achieved by use of the Rink amide resin to give the C-terminal amide (16% overall yield).[25] Switching the terminal pivalic acid capping moiety to acetic acid gave the final analogue \( \text{Bis}6 \), Table 1) for use in SAR studies.

The effects of \( \text{Bis}1-6 \) on the growth of the human colon cancer cell line, HCT-116, were probed using an IncuCyte Zoom platform. HCT-116 cells express an activating Ras mutation representing an aggressive and common form of human colon cancer.[26] The dual Src-Abi tyrosine kinase inhibitor dasatinib and the DMSO vehicle (0.1% v/v) were used in control assays. Confluence assays monitoring HCT-116 cell growth clearly indicated both a dose-dependent and time-dependent response for compounds \( \text{Bis}1-4 \), with \( \text{Bis}1 \) appearing to be the most active (Figure S1A). Apoptosis was measured dynamically using the caspase 3 biosensor NucView[27] and indicated that \( \text{Bis}1-4 \) exerted their cytotoxic activity through an apoptotic mechanism (Figure S1A). Compounds \( \text{Bis}5 \) and \( \text{Bis}6 \) showed little or no activity either in repressing cell growth, or inducing apoptosis. The relative activity of all the analogues was further evaluated by testing each Tz-BBA analogue as an 8-point half-log dose response in a 3 day alamarBlue® cell viability assay. Following 72 h incubation with each Tz-BBA analogue, cell viability was measured by fluorescent conversion of the alamarBlue® reagent and dose response curves were fitted to calculate the relative \( \text{EC}_{50} \) upon cell viability; compounds \( \text{Bis}1-4 \) showed nM \( \text{EC}_{50} \) activities whilst \( \text{Bis}5 \) and \( \text{Bis}6 \) showed no significant activity (Figure 1). These initial results indicated that substitution of either the N-terminal pivalate capping group, or \( \text{aa}^4 \) (the NMe-o-BrTyr residue) significantly reduces the activity of Tz-BBA analogues.

![Scheme 3. Semicarbazide resin synthesis and SPPS coupling. Reagents and Conditions: a) (i) CDI, DMF, rt, 3 h; (ii) BocNHNNH2, DMF, rt, 3 h; b) TFA:CH2Cl2 (1:1), rt, 1 h, followed by 2 x 10 min wash with 10% PrNHEt in DMF; c) AcOH:CH2Cl2 (2:98), rt, 16 h; d) TFA:CH2Cl2 (1:1), rt, 25 min; (ii) PrNHEt wash prior to coupling; e) (i) 2,6-lutidine, TMSOTf, CH2Cl2, 0 ºC – rt, 2 h; (ii) TBAF (1 M in THF), THF, rt, 20 min; f) Boc-protected \( \text{aa}^2, \text{aa}^3, \text{aa}^4, \text{aa}^5, \text{aa}^6 \); g) or pivalic acid, Oxyma, DIC, DMF, rt, 1 h; g) TFA:CH2O (4:1), rt, 1 h.](Image 305x274 to 548x414)

Table 1. Sequences of the Tz-BBA alanine scan analogues used to probe the structure activity relationships of BBA.

| N- \( \text{aa}^2 \) \( \text{aa}^3 \) \( \text{aa}^4 \) \( \text{aa}^5 \) \( \text{aa}^6 \) | \( \text{Opp} \) | \( \text{NMe}^– \) | \( \text{Me}^– \) | \( \text{O}^– \) | \( \text{NH}_2^– \) |
|---|---|---|---|---|---|
| \( \text{Bis}1 \) Piv Ala | MeMe-o-BrTyr | 4-Me-Pro-Tz | d-Leu | NMe-Phe | Opp |
| \( \text{Bis}2 \) Piv Ala | MeMe-o-BrTyr | 4-Me-Pro-Tz | d-Leu | NMe-Phe | Ala-NH2 |
| \( \text{Bis}3 \) Piv Ala | MeMe-o-BrTyr | 4-Me-Pro-Tz | d-Leu | Ala | Opp |
| \( \text{Bis}4 \) Piv Ala |Ala | 4-Me-Pro-Tz | Ala | NMe-Phe | Opp |
| \( \text{Bis}5 \) Piv Ala |Ala | 4-Me-Pro-Tz | d-Leu | NMe-Phe | Opp |
| \( \text{Bis}6 \) Ac |Ala | 4-Me-Pro-Tz | d-Leu | NMe-Phe | Opp |

![Figure 1. Cell viability for the human colon cancer cell line HCT-116. Dose response curves and calculated \( \text{EC}_{50} \) values determined by alamarBlue® cell viability assay after incubation of HCT116 cells with compounds \( \text{Bis}1-6 \) for 72 h (dose range: 0.003-10 μM). Conversion of the alamarBlue® reagent to the activated fluorescent resorufin cell viability indicator was calculated across DMSO (vehicle) and compound treated samples. Tz-BBA analogue molar concentration expressed on a log scale (X-axis). Dose response of compound treated cells expressed as a percentage of control (DMSO) values (Y-axis). Cell viability dose-response plots and \( \text{EC}_{50} \) values represent the mean from 3 independent experiments. Error bars: +/- Standard Deviation from \( n=3 \).](Image 548x414)

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To further investigate the mechanism of action of the active analogues (Bis1-3) Reverse Phase Protein Array (RPPA) profiling was conducted against a panel of 62 targets on a Zeptosens planar waveguide RPPA platform. The Zeptosens platform provides precise quantification of changes in the abundance of multiple protein and phosphorylated protein species in biological samples following exposure to compound dose- and time-series, thereby providing an unbiased evaluation of compound mechanism-of-action at the post-translational pathway level. The 62 pathway markers were selected to cover a broad range of key cancer driver pathways, canonical signaling nodes and the ERK and Akt signaling pathways previously demonstrated to be regulated by BBA in renal carcinoma cells (see Table S1 for antibodies and pathways studied). The only clearly discernable inhibition of pathway signaling was upon protein kinase C (PKC) and discernable reduction in total protein expression levels was of the insulin receptor substrate protein IRS-1 (see Figure S2). Both of which appeared to be more affected by analogues Bis1 and Bis2 than the other active analogue Bis3. In contrast to the results of previous studies performed on HeLa and renal carcinoma cells lines, for the HCT116 human colon cancer cell line RPPA analysis showed no inhibition of phosphorylation of MEK or Akt by any of the analogues tested (see Figure S2). IRS-1 is an intracellular signaling adaptor protein which can act as a docking site for SH2-containing proteins including PI3K and Grb2, thus linking it to the PI3K/Akt/mTOR and MAPK (ERK) pathways. However, IRS proteins can be phosphorylated on serine residues through negative feedback loops, which inhibits their function. Thus the expression of IRS-1, which itself varies from cell type to cell type, may not reflect the functional status of this adaptor protein. The relatively “clean” profile obtained by RPPA demonstrates that the Tz-BBA analogues do not result in global changes in stress pathways and canonical signaling events, and are suggestive of a selective mechanism-of-action.

Perhaps most striking in terms of defining a mechanism for the cytotoxicity were the morphological images which were collected using an ImageXpress high content microscope (Figure 2). All four active Tz-BBA analogues showed distinct morphological changes relative to the DMSO control (Figure 2a), but N-terminal analogues Bis5 and Bis6 gave rise to no significant changes (Figure 2f). The lead compound Bis1 and its C-terminal aa modified alanine analogue Bis2 show morphological changes including clear disruption and aggregation of F-actin filaments and reduced cell-substrate adhesion (Figure 2c,d) which are similar to those shown by compounds such as cytochalasin D (Figure 2b) and jasplakinolide which impair actin dynamics. The other analogues Bis3 and Bis4 show different morphological changes (Figure 2e), including more elongated cells with prominent F-actin filaments and promotion of cell-substrate adhesion; these changes either result from a different mechanism, or perhaps from the lower overall activity of these analogues. The morphological changes induced by Bis1 and Bis2 are in good accord with those previously reported for the parent compound BBA in HeLa cells, and indicate that the switch from thiazoline
to thiazole to facilitate the generation of these Tz-BBA analogues by SPPS has not impaired their activity.

Conclusions

Since its initial isolation in 2009, bisebromomamide (BBA) has been identified as targeting the ERK and Akt pathways in renal cell carcinoma cell lines,[7] and has been shown to be the first linear peptide actin-targeting molecule.[8] Through the generation of thiazoline analogues (Tz-BBA) we have been able to pursue an SPPS-based approach towards the investigation of its SAR for the first time, generating four analogues (Bis1-4) which showed nM cytotoxic activity against a human colon tumour cell line, HCT-116. Systematic alanine scanning has revealed that while the C-terminus may be altered without significantly affecting the activity of the resultant analogues; alteration of N-terminal residues (Bis5), or replacement of the N-terminal pivalate cap (Bis6) removes all activity. RPPA analysis suggests a very specific mode of action for the most active of these Tz-BBA analogues (Bis1 and Bis2) in HCT-116 cells, which results in reduced activity of PKC and reduced expression of IRS-1. This is particularly exciting since the oncogenic protein IRS-1 is over-expressed in a wide range of cancers.[31]

Two analogues (Bis1 and Bis2) have been shown to induce similar morphological changes in HCT-116 cells to those generated by the parent linear peptide in HeLa cells at comparable dosage levels; these changes are consistent with F-actin disruption and aggregation. Of these analogues, Ala-NH₂-terminated Tz-BBA derivative Bis2 is particularly attractive as a lead compound, since its SPPS synthesis can be conducted using high-yielding Rink amide resin chemistry, rather than the more-challenging semicarbazide-based resin synthesis which was employed in the construction of the other analogues. Thus our systematic SPPS-based approach towards the SAR analysis of this NRPS-derived linear peptide has allowed the identification of a new and promising lead for use in the design of anti-cancer therapeutics, or as a tool to study the role of IRS-1 expression and F-actin aggregation.

Experimental Section

Experimental procedures and spectroscopic data for the preparation of fragments Opp (3), 4-MeProTz (9) and Me₆-d-BrTyr (13) are presented in the Supporting Information.

SPPS Conditions for Semicarbazide Resin 14

Preparation of Resin 14: Aminomethyl polystyrene (300-500 mesh, 4.0 mmol/g) (0.20 g, 0.80 mmol, 1.00 eq.) was swollen in CH₂Cl₂ (5 mL) for 30 min; then washed with DMF (3 × 5 mL), CDI (0.65 g, 4.0 mmol, 5 eq.) in DMF (5 mL) was added to the resin and the resin was agitated for 3 h at rt. The resin was washed with DMF (3 × 5 mL), then tert-butyl carbazate (0.53 g, 4.00 mmol, 5 eq.) in DMF (5 mL) was added to the resin and the resin was agitated for a further 3 h at rt to give intermediate 15. The resin was washed with DMF (3 × 5 mL) and CH₂Cl₂ (3 × 5 mL), then a 1:1 mixture of TFA and CH₂Cl₂ (5 mL) was added to the resin and the resin was agitated for 1 h. The resin was washed with CH₂Cl₂ (3 × 5 mL), MeOH (3 × 5 mL) and CH₂Cl₂ (3 × 5 mL); then a solution of 10% DIPEA in DMF (3 mL) was added to the resin and the resin was agitated for 10 min at rt. This was repeated with fresh solution for a further 10 min, then the resin was washed with DMF (3 × 5 mL), CH₂Cl₂ (3 × 5 mL), MeOH (3 × 5 mL) and CH₂Cl₂ (3 × 5 mL). The TNBS test was performed and the deprotection was repeated if any colourless beads were observed. Semicarbazide resin 14 was used immediately.

Attachment of the Ketone-containing Opp residue: A solution of Boc-protected ketone 3 (2.4 mmol, 3.0 eq.) in anhydrous CH₂Cl₂ (5 mL) and 2% glacial acetic acid was added to the resin and the resin was agitated for 18 h at rt.

Amino Acid coupling: The Boc-protected amino acid residue (2.4 mmol, 3.0 eq.) and Oxyyma (0.34 g, 2.4 mmol, 3.0 eq.) were dissolved in DMF (3 mL) and DIC (0.37 mL, 2.4 mmol, 3.0 eq.) was added. The reaction mixture was agitated for 5 min before addition to the resin along with 1.5 eq. of 1,1′-EDT (0.21 mL, 1.2 mmol, 1.5 eq.), and the resin was then agitated at rt for 1 h. The reaction mixture was removed and the resin was washed with DMF (3 × 5 mL), CH₂Cl₂ (3 × 5 mL), MeOH (3 × 5 mL) and CH₂Cl₂ (3 × 5 mL). The TNBS test was performed and the deprotection was repeated if any colourless beads were observed.

Selective Boc deprotection: A 1:1 mixture of TFA and CH₂Cl₂ (5 mL) was added to the resin and the resin was agitated for 5 min. This was repeated with fresh solution for a further 20 min, then the resin was washed with CH₂Cl₂ (3 × 5 mL), MeOH (3 × 5 mL) and CH₂Cl₂ (3 × 5 mL). The TNBS test was performed and the deprotection was repeated if any colourless beads were observed.

Selective Boc deprotection: The resin was solvated in dry CH₂Cl₂ (8 mL) and chilled (dry ice) before addition of 2.6-lutidine (1.38 mL, 12.0 mmol, 15 eq.) and TMSOTf (1.74 mL, 9.60 mmol, 12 eq.). The resin was agitated at −78 °C for 15 min then at rt for a further 90 min. The reaction mixture was removed and the resin washed with CH₂Cl₂ (3 × 5 mL), MeOH (3 × 5 mL) and CH₂Cl₂ (3 × 5 mL). TBAF (4 mL, 1 M in THF) was then added and the resin was agitated for 10 min. This was repeated with fresh solution, then the resin was washed with CH₂Cl₂ (3 × 5 mL), MeOH (3 × 5 mL) and CH₂Cl₂ (3 × 5 mL).

Cleavage of Peptide from Semicarbazide Resin: A mixture of TFA:H₂O (5 mL:4:1) was added to the resin and the resin was agitated at rt for 1 h. The reaction solution was filtered and collected, then evaporated to dryness under nitrogen. The resulting residue was then purified by column chromatography (CH₂Cl₂:MeOH) to obtain the desired product.

Experimental procedures for the Rink amide coupling, together with purification procedures and spectroscopic data for peptides Bis1-6 are presented in the Supporting Information.

High-Content Imaging Methods

Kinetic cell growth and Apoptosis assays were performed using the IncuCyte-ZOOM® Live cell kinetic imaging system (Essen Bioscience). Cytoskeletal morphology assays were performed on the ImageXpress® High content imaging system (Molecular Devices).

Cell Viability Assay: Cells were seeded in a 96-well plate format at the appropriate cell concentration (5,000 cells/well) and incubated for 48 h before treatment. The media in each well was then replaced with fresh media containing each Tz-BBA analogue Bis1-6 as an 8-point half-log dose response and incubated for 3 days. alamarBlue® cell viability reagent (10% v/v) was added to each well and the plate was incubated for 1 h. Fluorescence emission was detected using a PerkinElmer Envision® multilabel reader (excitation filter at 540 nm and emissions filter at 590 nm). All conditions were normalized to the DMSO treated cells (100%) and curves fitted using GraphPad Prism using a sigmoidal variable slope curve. The data presented in Figure 1 represent the mean and standard deviation from 3 independent experiments.

Apoptosis Assay: Cells were seeded in a 96-well plate format at the appropriate cell concentration (5,000 cells/well) and incubated for 24 h before treatment. The media in each well was then replaced with fresh...
media containing each Tz-BBA analogue Bis1-6 as an 8-point half-log dose response and incubated for 5 days. Untreated cells were incubated with DMSO (0.1% v/v). Apoptosis was detected using the cell-permeable caspase biosensor NucView™ (Biotium) which was added to each well at 1 μM at the same time as compound treatment. The IncuCyte® imaging software automatically quantifies cell confluence and NucView™ Caspase positive objects at sequential time points following compound addition. This provided a kinetic read out of cell growth and apoptosis induction in cells following addition of each Tz-BBA analogue. Representative data and images from a least three independent experiments performed across separate weeks are presented in Figure S1.

**Cytoskeletal Morphology Assays:** Cells were seeded in a 96-well plate format at the appropriate cell concentration (5,000 cells/well) and incubated for 24 h before treatment. The media in each well was then replaced with fresh media containing each Tz-BBA analogue Bis1-6 as an 8-point half-log dose response and incubated for 48 h. Untreated cells were incubated with DMSO (0.1% v/v). All images were acquired on the automated ImageXpress microXL high-content imaging platform (Molecular Devices) using a x20 PanFluor ELWD Phl DM objective and a 16-bit camera (binning resolution of 1). Four separate fields of view per well were acquired using laser-based autofocus parameters optimized for cell plates and cell type. All immunostaining procedures were performed at room temperature in 96-well plates. All volumes are 100 μL unless otherwise stated. Cells were fixed following direct addition of 8% paraformaldehyde in PBS to cells (final concentration 4%) and were incubated for 20 min. Cells were washed three times with PBS then incubated with a mixture of Phalloidin conjugated to Alexa Fluor 546 (Molecular probes; diluted 1:500) and 4',6-diamidino-2-phenylindole (DAPI; Sigma D8417) for 45 min in the absence of light. Cells were washed three times with PBS again before imaging. Representative images are presented in Figure 2.

**Reverse Phase Protein Array Methods**

The abundance of total protein and phosphorylated protein epitopes was quantified using the ZepToReader reverse phase protein microarray platform as previously described. Briefly, HCT116 cells were seeded at 4 x 10^5 cells/well in 6-well plates. Cells were pre-incubated in treatment-free media for 48 h prior to addition of each Tz-BBA analogue Bis1-3 at 6-point half log dose response ranging from 3 – 0.03 μM for 30 min, 3 h and 24 h. Control samples were treated with media containing 0.1% (v/v) DMSO. Following exposure, the drug-containing medium was removed, and the cells were lysed with CLB1 buffer (Zeptosens, Bayer) for 30 min according to the manufacturer’s instructions. Cell lysates were normalized to a uniform protein concentration with spotting buffer CSBL1 (Zeptosens-Bayer) prior to preparing a final 4-fold concentration series of; 0.2; 0.15; 0.1 and 0.75mg/ml. The diluted concentration series of each sample was printed onto Zeptosens protein microarray chips (ZepToChip™, Zeptosens-Bayer) under environmentally controlled conditions (constant 50% humidity and 14 °C temperature) using a non-contact printhead (Pannoplotter 2.1e, GeSIM). A single 400 μl droplet of each lysate concentration was deposited onto the Zeptosens chips. A reference grid of AlexaFluor647 conjugate BSA consisting of 4 columns x 22 rows was spotted onto each sub-array, each sample concentration series were spotted in between reference columns. After array printing, the arrays were blocked with an aerosol of BSA solution using a custom designed nebulizer device (ZepToFog™, Zeptosens-Bayer) for 1 h. The protein array chips were subsequently washed in double-distilled water and dried prior to performing a dual antibody immunocassay. The arrays were incubated with a panel of 62 primary antibodies (Table S1) overnight at room temperature followed by 2.5 h incubation with secondary Alexa-Fluor conjugated antibody detection reagent (anti-rabbit A647 Fab, Invitrogen). Following secondary antibody incubation and a final wash step in BSA solution, the immunostained arrays were imaged using the ZeptoREADER™ instrument (Zeptosens-Bayer). For each-subarray, five separate images were acquired using different exposure times ranging from 0.5-10 s. Microarray images representing the longest exposure without saturation of fluorescent signal detection were automatically selected for analysis using the ZepToView™ 3.1 software. A weighted linear fit through the 4-fold concentration series was used to calculate relative fluorescence intensity (RFI) value for each sample replicate. Local normalization of sample signal to the reference BSA array was used to compensate for any intra- or inter-array/chip variation. The panel of 62 protein analytes (see Table S1) were normalized by the following global normalization procedure using the entire antibody panel: (a) Determine median for each antibody across the sample set. (b) Divide each raw linear value by the median within each antibody to obtain the median-centred ratio. (c) Calculate the median from median-centred ratio for each sample across the entire panel of antibodies. This median functions as a correction factor for protein loading adjustment. (d) Divide raw RFI data by the correction factor to obtain the normalized values. Global normalized data for each Tz-BBA analogue is subsequently normalized to DMSO control for each time point and plotted as bar graphs (see Figure S2).

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