SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Identification of a novel BUB1 inhibitor. (a) Overview of the primary HTS campaign. The left and middle panels show histograms with the respective statistical distributions of controls and test compounds. The right hand panel shows the data quality Z'-factor throughout the campaign. (b) Overview of the secondary HTS experiments. The top panel shows a correlation of the hit activity in the primary HTS (10 µM, n = 1) vs. the activity at the same concentration in retest (n = 4). Data highlighted in green represent the confirmed hits. The bottom panel shows the correlation of compound activity a 10 µM *versus* the IC_{50} values obtained in dose-response experiments. The green dot (~95% inhibition and IC_{50} ~0.6 µM) indicates the single hit which ultimately led to BAY 1816032. (c) Biochemical mode of action study of the resynthesized HTS hit using the biochemical TR-FRET assay described in the methods section. The top panel shows dose-response inhibition curves (in black) obtained at the ATP concentrations indicated in the legend without prior pre-incubation of the compound with BUB1. The red curve was also measured with 1 mM ATP but the compound and BUB1 were pre-incubated for 15 min before start of the enzymatic reaction. The bottom panel shows the fit of the IC_{50} values obtained at different ATP concentrations (without pre-incubation) to the Cheng-Prussof equation (1), resulting in the Ki value shown in Table1. (d) kPCA traces corresponding to the displacement of Invitrogen's Kinase Tracer 236 by increasing concentrations of the compound. Fitting of these curves to the Motulsky and Mahan competitive kinetics model (2) resulted in the affinity and rate constants shown in Table 1. (e) SPR single cycle kinetics sensorgram corresponding to the titration of the compound as indicated in the methods section.

Fitting this sensorgram to the Langmuir model yielded the affinity and rate constants shown in Table 1.

Supplementary Figure S2

BAY 1816032 does not inhibit ezrin/radixin/moesin phosphorylation and BUB1 kinase activity is not required for TGFß-induced SMAD2/3 phosphorylation. (a) Jeg-3 and HCT116 cells were incubated with erlotinib for 5 min or with BAY 1816032 for 5 and 60 min at 10 µM. Blots of cell lysates were immunoprobed for phospho-ezrin/radixin/moesin (pERM) and ezrin. (b) A549 cells were stimulated with TGFß (10 ng/mL) for 1 h in the presence of TGFRI-inhibitor SB-431542, 2OH-BNPP1, and the selective BUB1 inhibitors BAY 1816032 and BAY-320 as indicated. Blots of cell lysates were immunoprobed for phospho-SMAD2 (Ser465/467), SMAD2, phospho-SMAD3 (Ser423/425), SMAD3, and GAPDH.

Supplementary Figure S3

Cell cycle distribution upon single agent and combination treatments. (a) MDA-MB-468 cells were treated with vehicle (0.5% DMSO), 1 µM BAY 1816032, or 0.4 µM AZ20 as single agents or in combination. Left panel: Cells were analyzed for DNA content 24, 48 (n = 3) and 72 h (n = 4) after treatment by means of flow cytometry. Right panel: Representative cell cycle profiles taken after 72 h of compound exposure. (b) MDA-MB-436 cells were treated with vehicle (0.5% DMSO), 1 µM BAY 1816032, or 6 µM olaparib as single agents or in combination. Left panel: Cells were analyzed for DNA content 24, 48, and 72 h (n = 3) after treatment by means of flow cytometry. Right panel: Representative cell cycle profiles taken after 72 h of compound exposure.

SUPPLEMENTARY METHODS

Enzymatic Mode of Action and Ki determination

For mechanism-of-inhibition studies IC_{50} determinations were performed at increasing concentrations of ATP (0.1xKm, 1xKm, 10xKm, 100xKm and 1000xKm). To this end incubation times were adjusted to guarantee kinetic linearity at each ATP concentration analyzed. The apparent H2A peptide and ATP Km values for Bub1 were determined in separate experiments as described elsewhere (3). The Km for ATP determined was 3 µM, whereas in SPR the K_D for ATP binding was 14 µM. To calculate the Ki the IC_{50} values were plotted vs. [ATP] and fitted to the Cheng-Prusoff equation for competitive binding (1) as described in (4).

Equilibrium and Kinetic Probe Competition Assays (ePCA and kPCA)

In order to characterize the kinetics of the binding of BAY 1816032 and related compounds to BUB1, a TR-FRET kinetic probe competition assay (kPCA) (5) was developed using N-terminal biotinylated BUB1 catalytic domain (6, 7). Experiments were performed as described in (5) for CDK2, in the buffer used for Bub1 kinase activity assays (8). The final concentrations of BUB1 protein and Tracer 236 (Invitrogen) in the assay were 200 pM and 200 nM, respectively. Steady state affinity measurements (ePCA) were conducted as previously reported (7). The rate constants for the association (k_a or k_{on}) and dissociation (k_d or k_{off}) were calculated by fitting the kPCA traces to the Motulsky and Mahan equation for competitive binding kinetics (2). The steady state affinity (Ki) was obtained by analyzing ePCA curves with a logistic 4-parameter model including the Cheng-Prusoff relationship (1).

Surface Plasmon Resonance (SPR)

SPR experiments were done at Biaffin GmbH & Co KG on Biacore T100 and T200 Systems (GE Healthcare) using the Biotin CAPture kit and sensor chip (GE Healthcare). Biotinylated human Avi-tagged BUB1 (amino acids 704-1085) was diluted 20 µg/ml in running buffer [PBS (pH 7.4), 0.05% P20, 1 mM DTT, 50 µM EDTA, supplemented with 3% DMSO] and injected 3 min at 2 µl/min to reach a density of ~1-2 kRU. Compounds were serially diluted in DMSO (3.7-1000 or 37-3000 nM, 1:3 step), and transferred to assay buffer to achieve their final test concentrations at a DMSO concentration of 3%. For binding analysis, a single cycle kinetics protocol was chosen with contact times of 0.5-1 min and a dissociation time of 30 min. To obtain kinetic and affinity parameters, sensorgrams (acquired at 10 Hz) were fitted using the BIAevaluation Software (GE Healthcare) to a 1:1 Langmuir model accounting for mass transport limitations or –in

particular cases- to a two-state model. Steady state analysis was performed with the same software using a single site equilibrium binding equation.

Isothermal Titration Calorimetry (ITC)

Experiments were performed at 25°C in an ITC200 microcalorimeter (MicroCal, Inc., Northampton, MA). Compounds at concentrations of 10, 20 or 30 µM were titrated with 100 µM BUB1 in 50 mM Tris/HCl, pH 7.7, 100 mM KCl, 10% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP) using 20 injections of 0.2 µl within 0.4 s at 100-s intervals and 1000 rpm stirring speed. Reference Power was set to 6 µcal/s and the filter period to 2 s. Data were collected on high feedback mode. Separate blank titrations of compound into buffer were performed to correct for heats generated by dilution and mixing. Analysis was performed with a single binding site model in the Origin MicroCal analysis software (version 7 SR4, MicroCal, Inc.). Thermodynamic parameters were calculated using $\Delta G = \Delta H - T\Delta S = -RT\ln K_B$, where ΔG, ΔH and ΔS are the changes in free energy, enthalpy and entropy of binding respectively using a single binding site model. No more than 10% of data were excluded from analysis.

Cell proliferation assay

Cells were seeded into 96 well plates at densities ranging from 1,000 to 5,000 cells per well in the appropriate medium supplemented with 10% FCS. After 24 hours, cells were treated in quadruplicates with serial dilutions of compounds. After further 96 hours, adherent cells were fixed with glutaraldehyde and stained with crystal violet. IC_{50} values were calculated by means of a 4 parameter fit.

In vitro combination assay

BAY 1816032 (concentration range, 1E-07 M to 1E-05 M) combination studies were conducted with a second compound selected from paclitaxel (1.0E-09 M to 1.0E-07 M),

docetaxel (3.0E-10 M to 3.0E-08 M), cisplatin (1.0E-07 M to 1.0E-05 M), AZ-20 (3.0E-08 M to 3.0E-06 M), AZD6738 (3.0E-07 M to 3.0E-05 M), VX-970 (1.0E-07 M to 1.0E-05 M), BAY 1895344 (3.0E-09 M to 3.0E-07 M), olaparib (3.0E-07 M to 3.0E-05 M), rucaparib (3.0E-07 M to 3.0E-05 M), or talazoparib (3.0E-08 M to 3.0E-06 M). Cells were plated into 384-well plates at densities between 600 and 800 cells per well. After 24 h, cells were treated with BAY 1816032 and the second compound either as single compound treatments, or in nine fixed compound ratios. Cell viability was assessed after 96 hour exposure, using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). IC_{50} values were determined by means of a 4 parameter fit and isobolograms and combination indices (CI) were calculated according to the median-effect model of Chou-Talalay (9). A CI of ≤0.8 was defined as more than additive (i.e. synergistic) interaction, and a CI of ≥1.2 was defined as antagonistic interaction.

Animal efficacy studies

Housing and handling of animals was in strict compliance with European and German Guidelines for Laboratory Animal Welfare. For SUM-149 xenograft studies female athymic NMRI *nu/nu* mice (Taconic), 5-6 weeks old, average body weight 20-22 g, were used after an acclimatization period of 14 days. SUM-149 cells derived from exponentially growing cell cultures were re-suspended in 100% Matrigel (BD Biosciences) to a final concentration of 5 $x10^7$ cells /ml and implants of 0.1 ml (5 x 10^6 SUM-149 cells) were inoculated into the inguinal region of athymic mice. Female, 6 weeks old NOD-SCID mice (NOD.CB17-Prkdc^{scid}/J, Charles River, Iffa Credo), average body weight 20-23 g, were used for MDA-MB-436 xenograft studies after an acclimatization period of 7 days. Animals were injected with 20 µl $1x10^6$ MDA-MB-436 cells suspended in 75% PBS/25% Matrigel into the 4th mammary fad pad. Feeding and

drinking was *ad libitum* 24 hours per day. Tumor area (product of the longest diameter and its perpendicular), measured with a caliper, and body weight were determined two to three times a week. Tumor growth inhibition is presented as T/C ratio (Treatment / Control) calculated with tumor areas at study end. Animal body weight was used as a measure for treatment-related toxicity. Body weight loss >20% was dedicated as toxic. When tumors reached a size of approximately 20-40 mm², depending on growth of the tumor model, animals were randomized to treatment and control groups (12 mice / group) and treated p.o. with vehicle (90% polyethylene glycol 400, 10% Ethanol), BAY 1816032, and/or paclitaxel, docetaxel or olaparib (dissolved in 10% DMSO, 90% [10% HPbetaCD in PBS, pH7.8]) as indicated in tables and figure legends. In combination treatment groups the compounds were applied at the same day within a time frame of 1 hour. Treatment of each animal was based on individual body weight. Animals were euthanized according to the German Animal Welfare Guidelines. Data were expressed as means ± SD. Statistical analysis included one way analysis of variance, differences to the control were compared *versus* control group by pair-wise comparison procedure, and differences between treatment groups were compared by *t*-test procedure using the SigmaStat software.

Immunohistochemistry

Paraffin-embedded skin sections from compound-treated nude mice were treated for 17 min in Target Retrievel Solution pH 9 (Dako S2367) in a steam cooking device. Sections were then stained using the rabbit anti-phospho-threonine 120 histone H2A antibody (Assay Bio Tech A8247 (Lot 118247), 1:400) in DAKO antibody diluent (S3022) for 90 min at RT, and anti-rabbit Envision secondary antibody (DAKO K4010) for 30 min at RT and DAB-Subtrate (Dako K3468) for 10 min at RT. Stained slides were scanned

using a Mirax slide scanner and evaluated for positive pixels above background in three independent fields per slide.

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