**Supplementary methods for:**

**The novel ATR inhibitor BAY 1895344 is efficacious as monotherapy and combined with DNA damage-inducing or repair-compromising therapies in preclinical cancer models**

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**Supplementary Methods**

**Compounds**

BAY 1895344 (2-[(3R)-3-methylmorpholin-4-yl]-4-(1-methyl-1H-pyrazol-5-yl)-8-(1H-pyrazol-5-yl)-1,7-naphthyridine) was identified and synthesized at Bayer AG (Germany; Fig. 1) as described previously (BAY 1895344 is Example 111 in patent WO 2016/020320 (1). Ibrutinib, olaparib, rucaparib, niraparib, talazoparib, 5-FU, cisplatin and carboplatin were purchased from commercial providers. AZD6738 was synthesized at WuXi AppTec (China), and the analytical data of the delivered material were identical to the reported data for Example 2.02 in patent WO 2011/154737 (2). M6620 was purchased from abcr GmbH (Germany), and the analytical data of the delivered material were identical to the reported data for Example 57a (compound IIA-7) in patent WO 2010/071837 (3). Darolutamide was synthesized at Orion Corporation (Espoo, Finland). For *in vivo* studies, BAY 1895344 was formulated in a vehicle consisting of PEG 400 (polyethylene glycol 400), ethanol and water (60:10:30) at pH 7–8. For *in vitro* studies, BAY 1895344 was dissolved in 100% DMSO (dimethyl sulfoxide).

**Cloning of expression vector**

The cloning of expression vectors for recombinant, human glutathione S-transferase/ FLAG-tagged full-length ATR and for recombinant, human Strep II-tagged full-length ATR-interacting protein (ATRIP) was performed as follows. The cDNAs encoding the protein sequences of full-length human ATR sequence (Q13535) with anN‑terminally fused Flag-Tag, and the full-length human ATRIP (Q8WXE1) were optimized forexpression in eukaryotic cells and synthesized using the GeneArt technology at Life Technologies.Both cDNAs also encoded att-site sequences at the 5’ and 3’ ends, allowing subcloning into the followingdestination vectors using the Gateway Technology: pD-MamA (an in-house derivative of the vectorpEAK from EdgeBioSystems but with a human CMV promoter), which provides a N-terminal fusionof a GST-Tag to the integrated gene of interest, and pD-MamB (an in-house derivative of pTT5 fromNRCC, Y. Durocher), which provides a N-terminal fusion of a STREP II-Tag to the integrated gene.ATR cDNAs were cloned into pD-MamA and the ATRIP cDNA into pD-MamB.

**Co-expression of ATR and ATRIP by transient transfection in HEK293-6E cells**

For transient transfection of HEK293-6E suspension cells, a Biostat Cultibag Bioreactor with 5 Lculture volume (starting volume) in a 20 L culture bag was used. The cells were cultured in F17medium (ThermoFisher) supplemented with Pluronic F68 (10mL/L of 10% solution, ThermoFisher), Gluta-Max (20mL of 100x solution/L, containing 200mM L-alanyl-glutamine;Invitrogen), and G418 (25 μg/mL, ThermoFisher) at 37°C, pO2 55% with agitation (18 rpm), until thecells reached a density of 1.6 x 106 cells/mL (99% viability). The transfection solution was preparedby adding 4 mg ATR**-**encoding plasmid, 1 mg ATRIP-encoding plasmid and 10 mg PEI (polyethylenimine, linear,Polysciences) into 500 mL F17 medium (without supplements), carefully mixed andincubated for 15 min at room temperature. This transfection solution was added to the cell culture and incubated for 5 h at 37 °C, pO2 55% with agitation (18 rpm). Next, 5 L of F17medium with the abovementioned supplements was added and the rocking rate was increased to 19 rpm.Cells were harvested by centrifugation (30 min, 1000 x g, 15 °C) 48 h post transfection and stored at -80 °C.

**Purification**

Purification of the ATR (Flag-Tag)/ ATRIP (Strep-Tag) complex was achieved by affini­ty chromatography using anti-FLAG-resin (Sigma, A-2220). Cells were lysed in Buffer A (50 mM Tris-HCl [pH 7,5], 150 mM NaCl, 5% Glycerol, 1 mM Na3VO4, 1 mM NaF, 10 mM S-glycero­phosphate, 1% Tween20; 0.1% NP40; completed with EDTA [Roche, 1 tablet/ 50 mL buffer]) for 1 h at 4 °C. The lysate was then centrifuged at 20.000 x g and the super­natant was batch-bound to Flag-agarose beads, washed three times using Buffer B (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10% glycerol) and eluted using Buffer C (Buffer B +200 μg/mL FLAG® peptides (Sigma, F3290) Purified proteins were snap-frozen in liquid nitrogen and stored at -80 °C. ATR concentration in the final preparation was 250 μg/mL. The yield of the co-purified ATRIP was far below a 1:1 ratio com­pared to ATR, but essential for ATR activity.

**Affinity of BAY 1895344 to ATR**

A time-resolved fluorescence resonance energy transfer (TR-FRET)-based ATR competition binding assay was used to determine the affinity of BAY 1895344 to ATR using fluorescent 5-TAMRA-labelled Tracer A [(described in (4)]. BAY 1895344, was dissolved and diluted (as 100x final concentration) in DMSO, and 50 nL aliquots were transferred into a black small-volume 384-well microtiter plate (Greiner Bio-One, Germany) using a 384-well capillary pipettor (HummingbirdTM, Genomic Solutions, UK). ATR working solution was prepared by mixing 3 nM GST-ATR/ATRIP with terbium-labeled anti-GST antibody (anti-GST-Tb) and incubated for 30 min at 22 °C to allow anti-GST-Tb/GST-ATR complex formation. Then, 3 µl of the ATR working solution was added to the microtiter plate and incubated for 15 min at 22 °C to allow binding of BAY 1895344 to GST-ATR, before the addition of Tracer A (40 nM final concentration). The plate was incubated for at least 30 min at 22 °C. Time‑resolved fluorescence energy transfer was measured with a PHERAstar plate reader (BMG Labtechnologies, Germany) or a ViewLux microplate imager (PerkinElmer, Boston, MA, USA) (excitation 337–350 nm, emissions 570 nm and 545 nm). The ratio of the emissions at 570 nm and at 545 nm was used to evaluate the amount of the anti-GST‑ GST‑ATR ‑Tracer A complex. Ratiometric data (570 nm/ 545 nm) were normalized as follows: the positive control included ATR-working solution and Tracer A (= 0% inhibition), the negative control included all components except GST-ATR/ATRIP (= 100% inhibition). BAY 1895344 was tested in duplicates at 11 concentrations (20 µM, 5.7 µM, 1.6 µM, 0.47 µM, 0.13 µM, 38 nM, 11 nM, 3.1 nM, 0.89 nM, 0.25 nM and 0.073 nM). The IC50 value was calculated by four-parameter logistic fitting using the Screener software package (Genedata, Switzerland).

**Measurement of** γ**H2AX phosphorylation**

The ability of BAY 1895344 to inhibit intracellular ATR kinase activity was determined by measuring the level of phosphorylated histone protein H2AX (γH2AX) in hydroxyurea-treated HT-29, whereas activity on ATM kinase activity was determined in neocarzinostatin-treated M059J cells using an immunofluorescent assay. Briefly, HT-29 or M059J cells were plated in 96-well plates and cultured for 24 h. BAY 1895344 (final concentrations 3 nM – 10 μM; medium alone for controls) was added using a Tecan/HP D300 digital dispenser, followed by the addition of 100 μl hydroxyurea (final concentration 2.5 mM) or neocarzinostatin (final concentration 120 ng/mL) in growth medium, respectively. Samples were incubated for 30 min at 37 °C, then the growth medium was aspirated, and the cells were fixed by adding ice‑cold methanol for 15 min. After fixation, the cells were washed once with PBS, followed by incubation with Odyssey® blocking buffer (Li-Cor Biosciences Lincoln, NE, USA) for 1 h at 22 °C. Cells were then labeled with mouse anti-phospho-histone H2A.X (Ser139) antibody (1:500, clone JBW301, Merck Millipore, Germany) in blocking buffer for 1 h at 22 °C or overnight at 4 °C. Cells were washed three times with PBS and the secondary antibody, Alexa Fluor 488 (1:500, Fluor488‑linked anti-mouse-IgG antibody (donkey), ThermoFischer Scientific, Waltham, MA, USA), was applied for 1 h at 22 °C. Subsequently, cells were washed three times and fluorescence was read with an acumen® Explorer laser scanning cytometer (TTP Labtech, UK). The change (%) in the level of γH2AX was calculated by normalizing the measured values to the fluorescence values of untreated control wells (= 0%) and the fluorescence of the induced, DMSO-treated controls without test compounds (= 100%). Half-maximal γH2AX inhibition values (IC50) were determined by means of a four-parameter logistic fit.

**Measurement of AKT phosphorylation**

MCF7 cells were thawed, washed and resuspended in OptiMEM (Gibco) at a concentration of 1.0 x 106 cells/mL. First, 50 nL aliquots of the BAY 1895344 (final concentration 2.0 x 10-5 – 7.25 x 10-11 M, DMSO as control) were transferred to a 384-well microtiter plate using a Hummingbird liquid handler. Next, 3 μL of cell suspension (assay medium as control) was added to the wells using a Multidrop dispenser (ThermoFischer Scientific). Samples were incubated for 30 min at 37 °C / 5% CO2. For the detection and quantification of phosphorylated AKT (Ser473), a commercial HTRF detection kit (phospho-AKT (Ser473) assay kit, Cisbio, France) was used according to manufacturer’s instructions. Fluorescence (excitation 337 nm, emission 1620 nm, emission 2665 nm) was measured with a PHERAstar plate reader. The ratiometric data (665 nm/ 620 nm) were normalized to the vehicle (DMSO = 0% inhibition) and inhibitor (medium only = 100% inhibition) controls. Compounds were tested in duplicates at 11 concentrations (20 µM, 5.7 µM, 1.6 µM, 0.47 µM, 0.13 µM, 38 nM, 11 nM, 3.1 nM, 0.89 nM, 0.25 nM and 0.073 nM). IC50 values were calculated by four-parameter logistic fitting using the Screener software package (Genedata, Switzerland).

**Quantitative PCR analysis**

LAPC-4 cells were seeded in 6-well plates at a density of 600,000 cells/well in RPMI medium supplemented with 10% FBS (v/v). Cells were starved in RPMI medium supplemented with 10% charcoal-striped FBS (v/v) for 48 hours and then treated with different compounds. Androgen stimulation was performed using the synthetic androgen agonist R1881 (1 nM). For inhibitor treatments, the AR antagonist darolutamide (2 µM), the ATR inhibitor BAY 1895344 (75 nM), or a combination of both, was added to the wells. Cells were harvested 24 hours or 48 hours later. RNA was isolated using the RNeasy® Plus Mini kit (QIAGEN, Hilden, Germany) and reverse-transcribed to cDNA using the SuperScript® III First Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). Gene expression analysis was performed using the TaqMan® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) and the following TaqMan probes: BRCA1 (Hs01556193\_m1), EXO1 (Hs01116190\_m1) and MCM10 (Hs00218560\_m1), all from Applied Biosystems. All measurements were made in triplicate for each sample (n = 3). The data were analyzed according to the 2−ΔΔCt method using human cyclophilin A (PPIA Control Mix, Applied Biosystems) as a reference gene (relative expression to cyclophilin A). Statistical analysis was performed using Dunnett´s multiple comparisons test.

***In vitro* proliferation assay**

The anti-proliferative activity of BAY 1895344 was evaluated against a panel of 38 cancer cell lines (Table S3). Briefly, cells were plated in their appropriate growth medium in 96‑well plates, allowed to adhere for 24 h and incubated with different concentrations of BAY 1895344 (3 nM – 3 μM) for four days. Subsequently, the cells were fixed with glutaraldehyde, stained with crystal violet, and absorbance (595 nm) was read with a Tecan Sunrise absorbance microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Viability (corresponding to cell number) of non-adherent GRANTA‑519, Jeko-1, JVM‑2, REC-1 and SU-DHL-8 cells and adherent LAPC-4, C4-2B and VCaP cells was determined using the CellTiterGlo® (CTG) cell viability assay (Promega, Madison, WI, USA) according to manufacturer’s instructions. Briefly, cells were plated in their appropriate growth medium in microtiter plates, cultured for 24 h at 37 °C, followed by incubation with different concentrations of BAY 1895344 (3 nM – 3 μM) for 4 days (3 days for LNCaP C4-2B; 7 days for LAPC-4 and VCaP). Then CTG solution was added, and after 10 min, luminescence was measured with a VICTOR V multilabel plate reader (PerkinElmer).

All measurements were done in quadruplicates. Sample values were normalized to vehicle controls (solvent-treated cells = 100%) and to controls that were fixed at the time point of compound application (= 0%). Half-maximal growth inhibition (IC50) was determined by means of a four-parameter logistic fit. In the CTG assay, the change in cell viability was calculated by normalization with respect to the cell number at the beginning of the treatment and to the cell number of the untreated control group.

***In vitro* combination assays**

*In vitro* anti-proliferativeactivity of BAY 1895344 incombination with different chemotherapy drugs was assessed by determination of combination indexes in a panel of cancer cell lines. The combination of BAY 1895344 (3 – 300 nM) with cisplatin (100 nM – 10 μM) was investigated in HT-29, HeLa, HT-144 and NCI-H460 cells. The combination of BAY 1895344 (3 – 300 nM) with bleomycin (10 nM – 30 μM) or SN‑38 (3 – 300 nM) was investigated in HT-29, PC-3, HT-144, LOVO and MDA-MB-231 cells. Combination studies with BAY 1895344 (3 – 300 nM) and docetaxel (0.3 – 30 nM) were conducted in 22Rv1 cells. The combination of BAY 1895344 (3 nM – 1 μM) with AZD0156 (100 nM – 10 μM) or M9831 (VX-984, 300 nM – 30 μM) was investigated in HT-29, PC-3, HT-144 and LOVO cells. The combination of BAY 1895344 (3 nM – 1 μM) with AZD7762 (10 nM – 1 μM), PF 00477736 (10 nM – 1 μM) or AZD1775 (10 nM – 3 μM) was investigated in PC‑3 and LOVO cells. Combination studies with BAY 1895344 (3 nM – 1 μM) and MK‑8776 (100 nM – 10 μM) were conducted in LOVO cells. Cells were treated with a single compound or a combination of fixed compound ratios for four to six days and viability was measured using CellTiter-Glo®. EC50 values were calculated from triplicate values for each individual combination data point and the respective isobolograms were generated. A combination index (CI) of < 0.8 was defined as synergistic, 0.8 ≤ CI ≤ 1.2 as additive and a CI of > 1.2 was defined as antagonistic interaction.

**Plasma protein binding**

The plasma protein binding was determined using equilibrium dialysis by HTDialysis (Gales Ferry, USA) (5), in which a semi-permeable membrane separates the plasma and buffer compartments. BAY 1895344 (3 µM) was added to the plasma compartment and incubated under moderate agitation for 7 hours at 37°C (5% CO2, 99% humidity). Plasma samples were transferred into a deep-well plate, precipitated with 400 µl ice cold methanol and frozen over night at -20 °C. After thawing and mixing, the samples were centrifuged for 10 minutes at 3000 rpm, the supernatants were transferred into a 96-well plate and measured by LC-MS/MS. The unbound fraction (fu) of BAY 1895344 was determined as a ratio of unbound BAY 1895344 to the total concentration of BAY 1895344.

***In vitro* clonogenic combination assay**

The *in vitro* clonogenic combination assay was used to determine the survival of single cells and their ability to undergo unlimited division and grow into colonies after treatment with cytotoxic agents and radiation. LOVO cells were treated with the ATR inhibitor BAY 1895344 and radiation as monotherapy or in combination to evaluate the role of ATR inhibition in the radiosensitization of the cells. 70-80% confluent cells were treated with 3 nM BAY 1895344 for 24 hours and subsequently irradiated using the CellRad Faxitron at 150 kV, 5 mA, 2.3 Gy/min at shelf position 3. Two hours later, the cells were harvested, counted using a CASY cell counter (Schärfe System), re-plated into 6-well plates in sextuplicate (3 ml medium/well) and allowed to form colonies for 10-14 days and. Different cell concentration and radiation dose combinations were used:

|  |  |
| --- | --- |
| Radiation dose (Gy) | LOVO cells (cells/well) |
| 0 | 100 |
| 1 | 500 |
| 2 | 1000 |
| 3 | 5000 |
| 4 | 10000 |

Parallel samples were simultaneously washed, fixed with methanol/acetic acid (10:1) for 15 minutes at room temperature and stained for 20 minutes with 0.1% crystal violet staining solution (pH 4.5). Colonies were counted manually.

The plating efficiency (PE) and surviving fraction (SF) were determined as follows:

PE is the number of colonies formed divided by the number of seeded cells not exposed to radiation treatment.

$$PE=\left(\frac{number of colonies formed}{number of cells seeded}\right)∙100 \%$$

SF is the number of formed colonies after treatment of cells divided by the number of seeded cells with a correction for the PE. The SF at 0 Gy is set as “1” representing 100% cell survival.

$$SF=\frac{number of colonies formed after treatment}{number of cells seeded ∙\left(\frac{PE}{100 \%}\right)}$$

***In vivo* studies in cell line-derived xenograft (CDX) models**

The antitumor efficacy and tolerability of BAY 1895344 was evaluated in cell line-derived xenograft models in mice. Cancer cells from mid-log phase (70%) cultures were harvested and inoculated subcutaneously by injection of 100 µL cell suspension into the flank of mice, or orthotopically by injection of 20 µL cell suspension into the 4th mammary fat pad.

BAY 1895344 and AZD6738 were formulated in PEG400/EtOH/water (60:10:30), ibrutinib in 100% PEG400, M6620 in Solutol/EtOH/water (20:10:70), 5-fluorouracil (5-FU) and carboplatin in 0.9% NaCl, olaparib in 10% HPβCD ([2-hydroxypropyl]-β-cyclodextrin) in 0.9% NaCl and darolutamide in PEG400/propylene glycol/5% glucose (50:30:20).

When tumors reached a predetermined size of 25 – 50 mm2, mice were randomized into treatment and control groups (*n* = 10-11 mice/group), and treatments were started. The oral and intraperitoneal application volume was 10 mL/kg and the time interval between two applications per day was 6 – 7 hours.

Tumor response was assessed by measuring tumor area (length x width) using a caliper. Tumor area and body weight were determined 2 – 3 times per week. Changes in body weight throughout the study compared to initial body weight at treatment start were considered as a measure of treatment-related toxicity (>10% = critical, treatment on hold until recovery; >20% = toxic, termination). Mice were euthanized when showing signs of toxicity (>20% body weight loss), or when tumors reached a maximum size of 225 mm². Tumor growth inhibition is presented as T/C ratio (treatment/control), calculated using tumor area values at the end of the study. Relative tumor growth inhibition based on tumor area (T/Crel.area) was calculated using the following formula:

$$T/C=\left(\frac{\left[tumor area of treatment group at day x\right]-[tumor area of treatment group before treatment] }{\left[tumor area of control group at day x\right]-[(tumor area of control group before treatment]}\right)$$

Treatment responses were further assessed using RECIST criteria (7) and response rates were calculated accordingly (RR = number of mice with complete or partial response).

*In vivo* experiments with BAY 1895344 as monotherapy

2 x 106 of GRANTA-519 human mantle cell lymphoma cells suspended in 50% Matrigel® / 50% medium were inoculated subcutaneously into female SCID beige mice (5–6 weeks, 20–22 g, Envigo, France). To assess dose-dependent anti-tumor activity of BAY 1895344, mice were randomized into control and treatment groups (*n* = 9 mice/group) when GRANTA-519 tumors reached a size of 30 mm2, and treatment with vehicle (PEG400/EtOH/water 60:10:30, 2QD, 3 days on/ 4 days off, p.o.) or different doses of BAY 1895344 (3, 10, 30 or 50 mg/kg, 2QD, 3 days on/ 4 days off, p.o.) was started. All groups were sacrificed on day 30 after tumor inoculation. To evaluate the anti-tumor efficacy of BAY 1895344 in comparison to other ATR inhibitors, GRANTA-519 tumor-bearing mice were randomized into control and treatment groups (*n* = 8 mice/group) at a tumor size of 19-22 mm2, and treatment with vehicle (PEG400/EtOH/water 60:10:30, 2QD, 3 days on/ 4 days off, p.o.), BAY 1895344 (50 mg/kg, 2QD, 3 days on/ 4 days off, p.o.), AZD6738 (50 mg/kg, QD, p.o.) or M6620 (100 mg/kg, QD, p.o.) was started. All groups were sacrificed on day 29 after tumor inoculation.

1 x 106 of REC-1 human mantle cell lymphoma cells suspended in 100% Matrigel® were inoculated subcutaneously into female C.B‑17 SCID mice (5–6 weeks, 20 – 22 g, Taconic M&B A/S, Lille Skensved Ejby, Denmark). When tumors reached a size of 25–30 mm2, mice were randomized into treatment and control groups (*n* = 10 mice/group) and treatment with vehicle (PEG400/EtOH/water 60:10:30, 2QD, 3 days on/ 4 days off, p.o.), BAY 1895344 (50 mg/kg, 2QD, 3 days on/ 4 days off, p.o.), ibrutinib (20 mg/kg, QD, p.o.), AZD6738 (50 mg/kg, QD, p.o.) or M6620 (100 mg/kg, QD, p.o.) was started. Vehicle and BAY 1895344 treatment groups were sacrificed on day 28 or day 72 after tumor inoculation, respectively. Ibrutinib, AZD6738 and M6620 treatment groups were sacrificed on day 37 after tumor inoculation.

3 x 106 of PC-3 human prostate cancer cells suspended in 50% Matrigel® / 50% medium were inoculated subcutaneously into male NMRI nude mice (5–6 weeks, 20–22 g, Taconic). When tumors reached a size of 35–40 mm2, mice were randomized (*n* = 10 mice/group) and treatment with vehicle (PEG400/EtOH/water 60:10:30, 2QD, 3 days on/ 4 days off, p.o.), or BAY 1895344 (50 mg/kg, 2QD, 3 days on/ 4 days off, p.o.) was started. The groups were sacrificed on day 27 after tumor inoculation.

3 x 106 of LOVO human colorectal cancer cells suspended in 50% Matrigel® / 50% medium were inoculated subcutaneously into female NMRI nude mice (7 weeks, 20–22 g, Taconic). When tumors reached a size of 25–30 mm2, the mice were randomized (*n* = 10 mice/group) and treatment with vehicle (PEG400/EtOH/water 60:10:30, 2QD, 3 days on/ 4 days off, p.o.), BAY 1895344 (50 mg/kg, 2QD, 3 days on/ 4 days off, p.o.) or 5-FU (50 mg/kg, QW, i.p.) was started. The groups were sacrificed on day 28 after tumor inoculation.

2 x 106 of A2780 human ovarian cancer cells suspended in 100% Matrigel® were inoculated subcutaneously into female NMRI nude mice (5–6 weeks, 20–22 g, Janvier Labs, France). When tumors reached a size of 30–35 mm2, the mice were randomized (*n* = 10 mice/group) and treatment with vehicle (PEG400/EtOH/water 60:10:30, 2QD, 3 days on/ 4 days off, p.o.) or BAY 1895344 (50 mg/kg, 2QD, 3 days on/ 4 days off, p.o.) was started. The groups were sacrificed on day 15 after tumor inoculation.

*In vivo* mode-of-action (MoA) - γH2AX and pATR in GRANTA-519 tumor xenografts

Female SCID beige mice (5–6 weeks, 20–22 g, Envigo) were inoculated subcutaneously with 2 x 106 GRANTA-519 cells suspended in 50% Matrigel® / 50% medium. When tumors reached a size of 50–70 mm2, mice were randomized (*n =*16 mice/group) and treated with vehicle (PEG400/EtOH/water 60:10:30, 2QDx2 and QDx1, p.o.) or 50 mg/kg BAY 1895344 once (QDx1, p.o.) or repeatedly (2QDx2 and QDx1, p.o.). Mice were sacrificed, and tumors were collected at 3, 7, 24 and 48 hours after last treatment with BAY 1895344 (*n* = 4 mice/group at each time point). Tumor samples were lysed, and proteins were separated on SDS-PAGE followed by western blot analysis. The mouse anti-phospho-histone H2AX (Ser139) antibody (1:1000) was used for detection of human γH2AX. The GAPDH (14C10) rabbit monoclonal antibody (1:2000; Cell Signaling Technology, Danvers, MA, USA) was used to measure GAPDH as reference protein for signal quantification. For the detection of ATR phosphorylation (pATR) at threonine 1989, tumor lysates were separated by capillary electrophoresis and immunoprobed with an anti-phospho-ATR (Thr1989) antibody (GeneTex, GTX128145) followed by a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was quantified.

In a second experiment, the pharmacodynamic activity of different sub-MTD doses of BAY 1895344 was assessed. GRANTA-519 tumor-bearing mice were randomized into control and treatment groups (*n* = 21 mice/group) when tumors reached a size of 90 mm2, and treated with vehicle (PEG400/EtOH/water 60:10:30, 2QDx2 and QDx1, p.o.) or BAY 1895344 (3, 10, 30 mg/kg, 2QDx2 and QDx1 or 10, 30 mg/kg QDx1 = once, p.o.). Tumors were collected 0.5, 1, 3, 8, 24, 48 and 72 hours after the last treatment (*n* = 3 mice/group at each time point). For γH2AX determination, tumor samples were handled as described above.

In a third experiment, the kinetics of drug treatment effects on γH2AX levels were evaluated. GRANTA-519 tumor-bearing mice were randomized into control and treatment groups (*n* = 9 mice/group) when tumors reached a size of 30 mm2, and treated with vehicle (PEG400/EtOH/water 60:10:30, 2QDx2 and QDx1, p.o.) or BAY 1895344 (30 mg/kg, 2QD, 3 days on/ 4 days off, p.o.) for 1 cycle, 2 cycles or 3 cycles. Tumors were collected 8, 24 and 48 hours after the last treatment with BAY 1895344 (*n* = 3 mice/group at each time point). For γH2AX determination, tumor samples were analyzed as described above.

*In vivo* experiments with BAY 1895344 in combination with the chemotherapy drug carboplatin in the IGROV-1 human ovarian cancer model

Female nude (nu/nu) mice (5–6 weeks, 20–22 g, Taconic) were inoculated subcutaneously with 3 x 106 IGROV-1 human ovarian cancer cells in 100% Matrigel®. When tumors reached a size of 25-30 mm2, mice were randomized (*n* = 10 mice/group) and treatment with vehicle, BAY 1895344 (10 or 20 mg/kg, QD, 2 days on/ 5 days off, p.o.; or 50 mg/kg, 2QD, 3 days on/ 4 days off, p.o.) or carboplatin (50 mg/kg, QW, i.p.) as single agents and in combination was started. In combination groups (10 or 20 mg/kg BAY 1895344 and 50 mg/kg carboplatin), carboplatin was given 1–2 hours after treatment with BAY 1895344. All groups were sacrificed on day 28 after tumor inoculation.

*In vivo* experiments with BAY 1895344 in combination with radiation therapy (EBRT) in the LOVO colorectal cancer model

Female NMRI nude mice (5–6 weeks, 20–22 g, Taconic) were inoculated subcutaneously with 3 x 106 LOVO human colorectal cancer cells (in 50% Matrigel® / 50% medium). When tumors reached a size of 45–50 mm2, mice were randomized (*n* = 10 mice/group) and treatment with vehicle, BAY 1895344 (20 or 50 mg/kg, 2QD, 2 days on/ 5 days off, p.o.) or EBRT (QD) as monotherapies and in combination therapy was started. Radiation was applied on days 12 and 27 after tumor inoculation using a CP-160 X-ray irradiation system (Faxitron) at a dose rate of 0.65 Gray/min (5 Gray, 7.7 min), 160 kV, 6.2 mA, using a 0.5 mm copper filter. Animals were irradiated under anesthesia with 10 ml/kg Rompun/Ketavet (1:1 in 0.9% NaCl, i.p.). BAY 1895334 treatment was started on day 13 after tumor inoculation. The vehicle control group and BAY 1895344 20 mg/kg monotherapy group were terminated on day 39, BAY 1895344 50 mg/kg and radiation monotherapy groups on day 61 and both combination groups on day 74 after tumor inoculation.

*In vivo* experiments with BAY 1895344 in combination with the PARPi olaparib in the MDA-MB-436 human breast cancer model

Female NOD SCID mice (5–6 weeks, 20–22 g, Taconic) were inoculated with 1 x 106 MDA-MB-436 human breast cancer cells (in 25% Matrigel® / 75% PBS) orthotopically into the 4th mammary fat pad. When tumors reached a size of 30–35 mm2, mice were randomized (*n* = 10 mice/group) and treatment with vehicle, BAY 1895344 (20 or 50 mg/kg, 2QD 3 days on/ 4 days off, p.o.) or olaparib (50 mg/kg, QD, i.p.) as single agents and in combination was started. When combination partners were administered on the same day, olaparib was given 1–2 hours after the first treatment with BAY 1895344. All groups were sacrificed on day 83 after tumor inoculation.

*In vivo* experiments with BAY 1895344 in combination with the PARPi olaparib in the 22Rv1 human prostate cancer model

Male SCID mice (5–6 weeks, 20–22 g, Charles River Sulzfeld, Germany) were inoculated subcutaneously with 3 x 106 22Rv1 human prostate cancer cells (in 100% Matrigel®). When tumors reached a size of 34 mm2, mice were randomized (*n* = 10 mice/group) and treatment with vehicle, BAY 1895344 (20 mg/kg, 2QD 3 days on/ 4 days off, p.o.) or olaparib (20 or 50 mg/kg, QD, i.p.) as single agents and in combination was started. When combination partners were administered on the same day, olaparib was given 1–2 hours after the first treatment with BAY 1895344. All groups were sacrificed on day 24 after tumor inoculation.

*In vivo* experiments with BAY 1895344 in combination with the AR antagonist darolutamide in the LAPC-4 human prostate cancer model

Male C.B-17 SCID mice (11 weeks, 25 g, Janvier) were implanted with testosterone pellets (12.5 mg, 4 mm, prepared by Bayer AG), and three days later, inoculated subcutaneously with 2.5 x 106 LAPC-4 human prostate cancer cells in 100% Matrigel®. To assess the anti-tumor activity of BAY 1895344 in combination with darolutamide, mice were randomized into treatment and control groups at a tumor size of 50 mm2 (*n* = 11 mice/group) and treatment with vehicle, BAY 1895344 (20 mg/kg, 2QD, 3 days on/ 4 days off, p.o.) or darolutamide (100 mg/kg, QD, p.o.) as single agents and in combination was started. In addition, 11 mice were castrated and served as a control group in this hormone-dependent prostate tumor model. When the combination partners were administered on the same day, darolutamide was given 1–2 hours after the first treatment with BAY 1895344. The vehicle group and BAY 1895344 single agent group were sacrificed on day 51 and day 54 after tumor inoculation, respectively. All other groups were sacrificed on day 103 after tumor inoculation.

For the evaluation of the *in vivo* anti-tumor efficacy of the triple combination treatment with BAY 1895344, darolutamide and EBRT, LAPC-4 tumor-bearing mice were randomized into treatment and control groups when tumors reached a size of 50–60 mm2 (*n* = 10 mice/group). On day 26, treatments were started with EBRT (5 Gy, 7.7 min, QD, on day 26 and day 33 after tumor inoculation), followed by darolutamide (100 mg/kg, QD, p.o.) and subsequently with BAY 1895344 (20 mg/kg, QDx1, 2QDx2, QDx1 followed by 3 days off, p.o.) at least 2 hours after darolutamide treatment. After day 40 after tumor inoculation, no further radiation was applied and BAY 1895344 was administered at 2QD 3 days on/ 4 days off and darolutamide QD, as before. All treatments were applied in monotherapy, in all possible dual combinations and in triple combination in comparison to treatment with vehicle and castration control. The vehicle and BAY 1895344 monotherapy groups were sacrificed on day 53 after tumor inoculation. The darolutamide and radiation monotherapy groups as well as the group treated with the combination of BAY 1895344 and radiation were sacrificed on day 81 after tumor inoculation. All other groups were sacrificed on day 102 after tumor inoculation.

***In vivo* compound exposure**

To quantify the concentrations of ATR inhibitors in circulation, test substances were administered orally to mice (*n* = 2-3 for each compound and timepoint) in the respective studies in a solubilized form. Plasma samples were taken at the end of the study from sacrificed animals at multiple timepoints after the last substance administration (1, 3, 7, 24 hours). Samples were precipitated 1 : 5 (v:v) in ice-cold acetonitrile. After thawing and mixing, samples were centrifuged 2000 x g for 20 minutes at 4 °C and supernatants were analyzed by LC-MS/MS (AB Sciex, Framingham, MA, USA). Compound concentrations were determined using a calibration curve containing the same matrix as the injected sample and corrected for plasma protein binding. According to the free drug hypothesis (8), the unbound compound exposure in plasma is considered equal to the unbound compound concentration in the target tissue, therefore, the results can be put into relation to pharmacological potencies (here: IC50u) measured *in vitro*.

**Statistical analyses**

All analyses were performed using the statistical programming language R (version 3.5.2). The validity of the model assumptions was checked for each fitted statistical model. For *in vivo* studies presented in Fig. 2 (monotherapy) and 3 (mode of action), statistical analysis was performed using a linear model estimated with generalized least squares that included separate variance parameters for each study group to account for possible heteroscedasticity. Mean comparisons between the treatment and control groups were performed using the estimated linear model and corrected for family-wise error rate using Tukey’s or Sidak’s method. For *in vivo* studies presented in Fig. 1, 4, 5 and 6 (dose-response and combination studies), survival analysis was performed using the Cox proportional-hazards model. Longitudinal data was modelled using second order polynomial curves with random intercepts and slopes for each subject. Comparisons against the control group were adjusted for family-wise error rate using Dunnett’s method and all pairwise comparisons using Tukey’s or Sidak’s method. All longitudinal models included the first and second order fixed time effects for each group and random intercepts and slopes for each subject. Synergy was defined according to the Bliss Independence Model in the analysis of treatment combinations (9,10). Dose-response analysis was performed using 3-parameter logistic model that allows the top asymptote, ED50 and slope of the curve to vary based on the data, while the bottom asymptote is fixed at 0. Both 4- and 5-parameter curves were tested as well but the 3-parameter model was chosen based on the AIC (Akaike Information Criterion).

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