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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ABSTRACT

The DNA damage response (DDR) secures the integrity of the genome of eukaryotic cells. DDR deficiencies can promote tumorigenesis but concurrently may increase dependence on alternative repair pathways. The ataxia telangiectasia and Rad3-related (ATR) kinase plays a central role in the DDR by activating essential signaling pathways of DNA damage repair. Here, we studied the effect of the novel selective ATR kinase inhibitor BAY 1895344 on tumor cell growth and viability. Potent antiproliferative activity was demonstrated in a broad spectrum of human tumor cell lines. BAY 1895344 exhibited strong monotherapy efficacy in cancer xenograft models that carry DNA damage repair deficiencies. The combination of BAY 1895344 with DNA damage–inducing chemotherapy or external beam radiotherapy (EBRT) showed synergistic antitumor activity. Combination treatment with BAY 1895344 and DDR inhibitors achieved strong synergistic antiproliferative activity in vitro, and combined inhibition of ATR and PARP signaling using olaparib demonstrated synergistic antitumor activity in vivo. Furthermore, the combination of BAY 1895344 with the novel, nonsteroidal androgen receptor antagonist darolutamide resulted in significantly improved antitumor efficacy compared with respective single-agent treatments in hormone-dependent prostate cancer, and addition of EBRT resulted in even further enhanced antitumor efficacy. Thus, the ATR inhibitor BAY 1895344 may provide new therapeutic options for the treatment of cancers with certain DDR deficiencies in monotherapy and in combination with DNA damage–inducing or DNA repair–compromising cancer therapies by improving their efficacy.

Introduction

The DNA damage response (DDR) consists of multiple and diverse signaling pathways that secure the integrity of the genome of living cells (1). Proteins that directly recognize aberrant DNA structures recruit and activate kinases of the DDR pathways, which respond to a broad spectrum of DNA damage and to increased replication stress, for example, in oncogene-driven cancer cells (2–5). Many cancers harbor defects in DDR pathways, leading to genomic instability that can promote tumorigenesis and cancer cell growth. Concurrently, the defects in some signaling pathways may increase the dependence of cells on alternative repair pathways.

The ataxia telangiectasia and Rad3-related (ATR) kinase belongs to the phosphoinositide 3 kinase-related kinase family and plays a key role in the DNA replication stress response (RSR) pathway of DNA damage repair to maintain the genomic integrity through DDR activation (6–9). ATR is indispensable for cell survival by suppressing replication origin firing, promoting deoxyribonucleotide synthesis, and preventing DNA double-strand break (DSB) formation—ultimately averting mitotic catastrophe via induction of cell-cycle arrest and DNA damage repair (10). Therefore, loss-of-function mutations in the ATR pathway are very rarely observed, indicating essentiality for cellular survival (11). ATR is closely related to two other DDR kinases, ataxia telangiectasia mutated (ATM), and DNA-dependent protein kinase (DNA-PK). Together, these kinases build the core of the DDR by responding to different DNA damage insults, which are primarily DSBs for ATM and DNA-PK, and replication stress (RS) for ATR (12, 13). The ATM gene is frequently mutated in tumor cells, suggesting that loss of ATM activity is beneficial for cancer cell survival (11, 14). ATM kinase inactivation leads to increased dependence on ATR signaling, and combined inactivation of ATM and ATR induces synthetic lethality in cancer cells (15, 16).

The successful use of PARP inhibitors in patients with ovarian and breast cancer has shown that the blockade of selected DDR components is an effective strategy to treat cancers with specific types of DNA-repair deficiencies (17). Previous studies have demonstrated that ATR inhibition is also effective for treating cancers with defective DDR, such as homologous recombination (HR) deficiencies (18). In particular, cancer cells with high levels of oncogene-driven RS, for example, due to RAS mutation or MYC amplification, rely on ATR activity for survival, indicating the central role of ATR in response to RS (19). These findings demonstrate the potential of ATR inhibition to mediate synthetic lethality in cancer cells with increased RS or DDR defects.

The cancer-killing activity resulting from DNA-damaging agents such as irradiation or chemotherapy or DNA damage...
repair–compromising therapies such as PARP inhibitors (PARPi) or antiandrogen therapy may be increased by the pharmacologic inactivation of ATR, as indicated in previous preclinical studies (7, 20–26). Overall, there is strong potential for ATR inhibition as potent anti-cancer therapy in tumors characterized by increased DNA damage, deficiency in DDR or increased RS (7).

Here, we disclose the structure and functional characterization of the novel potent and selective ATR inhibitor (ATRi) BAY 1895344, which exhibits strong monotherapy efficacy in cancers with certain DDR deficiencies and synergistic activity in combination with DNA damage–inducing or DNA repair–compromising therapies, such as chemotherapy, external beam radiotherapy (EBRT), inhibition of PARP, or antiandrogen treatment.

Materials and Methods

Detailed descriptions of compounds and methods are presented in the Supplementary Materials and 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 (Fig. 1A) as described previously (BAY 1895344 is Example 111 in patent WO 2016/020320; ref. 27). Ibrutinib, olaparib, rucaparib, niraparib, talazoparib, 5-FU, cisplatin, and carboplatin were purchased from commercial providers. AZD6738 was synthesized at WuXi AppTec, and the analytic data of the delivered material were identical with the reported data for Example 2.02 in patent WO 2011/154737 (28). For chemical structure and additional information about AZD6738, see ref. 29. M6620 was purchased from abcr GmbH, and the analytic data of the delivered material were identical with the reported data for Example 57a (compound IA–7) in patent WO 2010/071837 (30). For chemical structure and additional information about M6620, see ref. 31. Darolutamid was synthesized at Orion Corporation.

Cell lines and culture conditions

Cancer cell lines were cultivated according to manufacturer’s (Supplementary Table S3) protocols at 37°C in a humidified atmosphere containing 5% CO2. Cell lines were regularly subjected to DNA contamination using MycoAlert (Lonza) directly before use. For experiments, cells from passages 3 to 10 were used, and for in vivo experiments, cells from passages 3 to 10 were used for inoculation.

Affinity and selectivity of BAY 1895344

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-labeled Tracer 1, an ATP-competitive ATRi (synthesized at Bayer AG; ref. 27). The ratio of the emissions at 570 and 545 nm was used to evaluate the binding affinity of BAY 1895344 to ATR.

The selectivity of BAY 1895344 was assessed using both an in-house kinase panel and a KINOMEscan Assay Panel (DiscoverX) consisting of 468 kinases, as described previously (32). In vitro experiments

The activity of ATR and ATM kinases was determined by measuring phospho-Ser139 histone protein H2AX (γH2AX) levels in hydroxyurea-treated HT-29 cells and neocarzinostatin-treated M059J cells, respectively. PI3K/AKT/mTOR signaling pathway activity was investigated in MCF7 breast cancer cells by measuring AKT phosphorylation.

The antiproliferative activity of BAY 1895344 was evaluated against a panel of 38 cancer cell lines (Supplementary Table S3). Cell proliferation was measured after 72 to 96 hours of exposure to BAY 1895344. Cell viability was determined using crystal violet staining or the CellTiter-Glo Cell Viability Assay (Promega).

The antiproliferative activity of BAY 1895344 in combination with different drugs was assessed by determination of combination indexes (CI). The combination of BAY 1895344 (3–300 nmol/L) with cisplatin (100 nmol/L–10 nmol/L) was investigated in HT-29 cells, and the combination with olaparib (300 nmol/L–30 μmol/L), niraparib (30 nmol/L–3 μmol/L), rucaparib (300 nmol/L–30 μmol/L), or talazoparib (1–100 nmol/L) in MDA-MB-436 cells. Combination studies with BAY 1895344 (10 nmol/L–10 μmol/L) and darolutamide (10 nmol/L–10 μmol/L) were conducted in LAPC-4 cells, in the presence of the synthetic androgen methyltrienolone R1881 (10 nmol/L). Additional combination studies with BAY 1895344 and a selection of compounds were conducted in a panel of cancer cell lines (Supplementary Table S4). Cells were treated with a single compound or a combination of fixed compound ratios for 4 to 6 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. CIs were calculated according to the median-effect model (33). 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.

The clonogenic combination assay (34) was used to assess the radiosensitization potential of BAY 1895344. LOVO colorectal cancer cells were treated with 3 nmol/L BAY 1895344 and different intensities of γ-radiation, allowed to form colonies for 10 to 14 days and, finally, the colonies were counted to calculate the combination effect.

In vivo studies in CDX models

All animal experiments were conducted in accordance with the German Animal Welfare Act and approved by local authorities. The in vivo antitumor efficacy and tolerability of BAY 1895344 as monotherapy/combination therapy were evaluated in CDX subcutaneous or orthotopic xenograft models in mice. Monotherapy experiments were performed in GRANTA-519 (in female SCID beige mice), REC-1 (in female C.B-17 SCID mice), PC-3 (in male NMRI nude mice), LOVO, and A2780 (both in female NMRI nude mice) models treated with BAY 1895344 at 50 mg/kg [all models; twice daily, 3 days on/4 days off (3on/4off), per os/orally] or at 3, 10, or 30 mg/kg (GRANTA-519; twice daily, 3on/4off, per os/orally), Ibrutinib (REC-1; 20 mg/kg, once daily, per os/orally), or 5-FU (LOVO; 50 mg/kg, once weekly, intraperitoneally). The combination of BAY 1895344 at 10 or 20 mg/kg [once daily, 2 days on/5 days off (2on/5off), per os/orally] or 50 mg/kg (twice daily, 3on/4off, per os/orally) and carboplatin (50 mg/kg, once weekly, intraperitoneally) was investigated in IGROV-1 tumor-bearing female nude (nu/nu) mice. The combination of 20 or 50 mg/kg BAY 1895344 (twice daily, 2on/5off, per os/orally) and EBRT (5 Gy; 7.7 minutes, once daily on days 12 and 27) was investigated in LOVO tumor-bearing female NMRI nude mice. Combination therapy experiments with 20 or 50 mg/kg BAY 1895344 (twice daily, 3on/4off, per os/orally) and 20 or 50 mg/kg olaparib (once daily, intraperitoneally) were performed in MDA-MB-436 and 22Rv1 models in...
Figure 1.
Dose-dependent antitumor efficacy and unbound plasma levels of BAY 1895344 as monotherapy. A, Chemical structure of BAY 1895344, a highly potent and selective inhibitor of ATR. B, Growth curves of GRANTA-519 mantle cell lymphoma tumors in female SCID beige mice (n = 10/group) treated with vehicle or different doses of BAY 1895344 (3, 10, 30, or 50 mg/kg, twice daily 3on/4off, per os/orally). C, Growth curves of GRANTA-519 tumors in female SCID beige mice (n = 10/group) treated with BAY 1895344 (50 mg/kg, twice daily 3on/4off, per os/orally = MTD), AZD6738 (50 mg/kg, once daily, per os/orally = MTD), or M6620 (100 mg/kg, once daily, per os/orally = MTD). D, Unbound BAY 1895344 plasma levels in relation to the determined biochemical, cellular mechanistic, and antiproliferative IC50 values of BAY 1895344 after repeated dosing with 3, 10, 30, or 50 mg/kg (twice daily, 3on/4off, per os/orally). E, Unbound BAY 1895344 plasma levels in relation to the respective in vitro antiproliferative IC50 values after repeated dosing with the ATRs BAY 1895344 (50 mg/kg, twice daily 3on/4off, per os/orally), AZD6738 (50 mg/kg, once daily, per os/orally), and M6620 (100 mg/kg, once daily, per os/orally) in GRANTA-519 tumor-bearing female SCID beige mice. Symbols refer to the individual measured concentrations after the final single dose on day 29 after tumor inoculation (end of last cycle); the dashed line represents the simulated concentration for 24 hours during twice daily dosing. Antiproliferative IC50 values for BAY 1895344, AZD6738, and M6620 in GRANTA-519 cells are 30, 1000, and 75 nmol/L, respectively. Relative tumor area (T/Crel.area) is defined as the percentage of the final tumor area on the day of vehicle group termination versus the initial tumor area (at the time point of starting treatment) of each individual mouse. Survival analysis was performed using the Cox proportional hazards model, and longitudinal data were modeled using second-order polynomial curves with random intercepts and slopes for each subject. Finally, dose–response analysis was performed using the three-parameter logistic model. **, P < 0.01 versus vehicle; ***, P < 0.001 versus vehicle; ****, P < 0.001 versus M6620. p.o., per os, orally; 2 QD, twice daily.
female NOD/SCID and male SCID mice, respectively. Combination experiments with 20 mg/kg BAY 1895344 (twice daily, 30/40 perf, per os/orally) and 100 mg/kg darolutamide (once daily, per os/orally) were performed in the hormone-dependent LAPC-4 prostate cancer model in male C.B-17 SCID mice. Castrated mice served here as a control. For a triple combination treatment, mice received EBRT (5 Gy, every 7 days twice) in addition to treatment with BAY 1895344 and darolutamide.

To elucidate the in vivo mode of action of BAY 1895344, ATR and H2AX phosphorylation was determined in lysed GRANTA-519 xenograft tumor samples. For the quantification of circulating ATRis, plasma samples were taken from mice and measured by LC-MS/MS.

Statistical analysis

Statistical analysis was performed using a linear model estimated with generalized least squares that included separate variance parameters for each study group (Figs. 2 and 3). Survival analysis was performed using the Cox proportional hazards model, and longitudinal data were modeled using second-order polynomial curves with random intercepts and slopes for each subject. Synergy was defined according to the Bliss Independence Model (35, 36) in the analysis of treatment combinations (Figs. 1, 4–6). Finally, dose–response analysis was performed using the three-parameter logistic model (Fig. 1).

Results

BAY 1895344 is a highly selective and potent inhibitor of ATR

BAY 1895344 (2-[(3R)-3-methylmorpholin-4-yl]-4-(1-methyl-1H-1,2,3-triazol-5-yl)-8-(1-methyl-5-yl)-1,7-naphthyridine) was discovered in a high-throughput screening approach (Fig. 1). The binding affinity of BAY 1895344 to its target molecule ATR was assessed by its ability to displace a fluorescently labeled ATR-binding tracer from a GST–ATR fusion protein using TR-FRET analysis. The IC_{50} of BAY 1895344 required to displace the tracer was 7.0 ± 3.7 nmol/L, indicating a high affinity of BAY 1895344 to ATR. The selectivity of BAY 1895344, evaluated in an in-house kinase selectivity panel and KINOMEScan profiling, demonstrated high selectivity of BAY 1895344 for ATR (Supplementary Tables S1 and S2). Only for one kinase out of 31 kinases in the in-house panel, mTOR, the determined IC_{50} of 35 ± 32 nmol/L was of the same order of magnitude as for ATR. Five other kinases were inhibited with an IC_{50} value in the concentration range tested (<20 µmol/L) and were at least 47-fold less potent compared with ATR (Supplementary Table S1). In the KINOMEScan panel, 1 µmol/L BAY 1895344 exhibited activity against only six of 456 kinases (Supplementary Table S2), and for only three of these kinases K_{D} values below 1 µmol/L were determined [mTOR, cyclin G-associated kinase (GAK) and right open reading frame kinase 2 (RIOK2), K_{D} = 24, 580, and 660 nmol/L, respectively]. Taken together, these results demonstrated the high selectivity of BAY 1895344 for ATR.

BAY 1895344 potently and selectively inhibits ATR-mediated DDR

Phospho-Ser139 histone protein H2AX (γH2AX) is a well-known early marker for DNA damage or DDR activation. Here, the activity of BAY 1895344 against ATR and ATM was evaluated in a downstream cellular mechanistic assay by determining the level of γH2AX. In HT-29 cells, BAY 1895344 inhibited hydroxyurea-induced ATR-dependent H2AX phosphorylation with an IC_{50} of 36 ± 5 nmol/L. In neocarzinostatin-treated DNA-PK−/− deficient M059J cells, BAY 1895344 had no effect on ATM-dependent H2AX phosphorylation even at 10 µmol/L, demonstrating that it is a potent and selective inhibitor of ATR kinase activity.

ATR and mTOR are PI3K-related protein kinases. AKT phosphorylation (pAKT) was used to monitor BAY 1895344-mediated mTOR inhibition in MCF7 cells. BAY 1895344 inhibited pAKT with an IC_{50} of 2.2 ± 0.9 µmol/L, whereas two mTOR inhibitors, PI-103 and AZD8055, inhibited pAKT with IC_{50} values of 71 ± 31 nmol/L and 12 ± 4 nmol/L, respectively. Hence, BAY 1895344 was 30- to 180-fold less active than specific mTOR inhibitors and showed superior selectivity for ATR compared with the PI3K/AKT/mTOR pathway (>60-fold).

BAY 1895344 inhibits proliferation in a broad spectrum of human cancer cell lines

In a panel of cancer cell lines of different histologic origins harboring various mutations affecting DDR pathways, BAY 1895344 showed potent antiproliferative activity with IC_{50} values ranging from 9 to 490 nmol/L (Supplementary Fig. S1; Supplementary Table S3). A clear difference between highly sensitive (IC_{50} < 100 nmol/L) and less sensitive cell lines was observed, and a majority of the sensitive cell lines were found to carry mutations affecting the ATM pathway independent of their origin (Supplementary Fig. S1A). Lymphoma appeared to be the most sensitive cancer type with IC_{50} values ranging from 9 to 32 nmol/L in mantle cell lymphoma cell lines (Supplementary Fig. S1B).

BAY 1895344 shows strong antitumor efficacy as monotherapy in CDX models

Antitumor efficacy of BAY 1895344 was evaluated in CDX models in mice. Dose-dependent efficacy was observed in the GRANTA-519 mantle cell lymphoma model, and the MTD and optimal dosing schedule of BAY 1895344 were identified as 50 mg/kg applied twice daily for 30/40 perf (Fig. 1B). In the GRANTA-519 model, treatment with BAY 1895344 applied at MTD demonstrated strong antitumor efficacy, whereas the ATR inhibitors AZD6738 and M6620 applied at MTD achieved only moderate antitumor efficacy (Fig. 1C).

When BAY 1895344 was dosed repeatedly (twice daily) at 30 or 50 mg/kg to GRANTA-519 tumor–bearing mice, the level of unbound BAY 1895344 in plasma covered the biochemical, cellular mechanistic, and antiproliferative IC_{50} concentrations determined in vitro, which is in line with the observed strong in vivo antitumor efficacy (Fig. 1D). Even at the highest applied dose (MTD), the level of unbound BAY 1895344 (1.6 µmol/L) was not sufficient to cover the IC_{50} concentration of 2.2 µmol/L required for the cellular inhibition of the PI3K/AKT/mTOR signaling pathway, suggesting that the antitumor activity of BAY 1895344 was not mediated by mTOR pathway inhibition. The level of unbound AZD6738 or M6620 in plasma did not exceed the antiproliferative IC_{50} concentrations, indicating that exposure to these ATRis may not have been sufficient to mediate antitumor activity in vivo (Fig. 1E).

Antitumor effects of BAY 1895344 as a single agent was further evaluated in CDX models of different indications characterized by DDR defects or oncogenes potentially mediating R5 (Supplementary Table S3). BAY 1895344 applied at MTD resulted in strong antitumor efficacy as demonstrated in the A2780 ovarian cancer, PC-3 prostate cancer, LOVO colorectal cancer, and REC-1 mantle cell lymphoma models (Fig. 2). The efficacy of BAY 1895344 clearly exceeded the efficacy of other treatments, as 5-FU (5-fluorouracil) failed to inhibit LOVO tumor growth, and AZD6738, M6620, and the BTK inhibitor ibrutinib showed significantly weaker efficacy (P < 0.001) in the REC-1 model.
Antitumor efficacy of BAY 1895344 as monotherapy. The optimal dose and treatment schedule for BAY 1895344 was 50 mg/kg (twice daily 3on/4off, per os/orally), based on efficacy and tolerability in the GRANTA-519 human mantle cell lymphoma xenograft model in female SCID beige mice. 5-FU, ibrutinib, AZD6738, and M6620 were used at their known MTDs on the basis of published data (39, 44). All treatments were well tolerated without toxicities (no death, acceptable body weight loss).

**A**, Growth curves of A2780 human ovarian tumors in female NMRI nude mice (n = 10/group) treated with vehicle or BAY 1895344. **B**, Relative tumor areas (T/Crel.area) of the A2780 tumors shown in A at study end. **C**, Growth curves of PC-3 human prostate tumors in male NMRI nude mice (n = 10/group) treated with vehicle or BAY 1895344. **D**, Relative tumor areas of the PC-3 tumors shown in C at study end. **E**, Growth curves of LOVO human colorectal tumors in female NMRI nude mice (n = 10/group) treated with vehicle, BAY 1895344, or 5-FU (50 mg/kg, once weekly, intraperitoneally). **F**, Relative tumor areas of the LOVO tumors shown in E at study end. **G**, Growth curves of REC-1 human mantle cell lymphoma tumors in female C.B-17 SCID mice (n = 10/group) treated with vehicle, BAY 1895344, ibrutinib (20 mg/kg, once daily, per os/orally), AZD6738 (50 mg/kg, once daily, per os/orally), or M6620 (100 mg/kg, once daily, per os/orally). **H**, Relative tumor areas of the REC-1 tumors shown in G on day 28 after tumor inoculation. Relative tumor area is defined as the percentage of the final tumor area on day of control termination versus the initial tumor area (at the time point of starting treatment) of each individual mouse. Statistical analysis was performed using a linear model estimated with generalized least squares that included separate variance parameters for each study group. **⁎**, P < 0.001 versus vehicle; 5-FU, 5-fluorouracil; i.p., intraperitoneally; PD, progressive disease; p.o., per os/orally; PR, partial response; QD, once daily; 2 QD, twice daily; QW, once weekly; SD, stable disease.
model. All treatments with BAY 1895344 were well tolerated over a treatment time of 11 to 59 days without toxicities and acceptable body weight loss (10%–13%; Supplementary Fig. S2).

To analyze the association between in vivo antitumor efficacy and the inhibition of ATR, ATR phosphorylation (pATR) as a direct measure of ATR kinase activity (37) and H2AX phosphorylation as an indirect measure of ATR-mediated DNA repair were determined in GRANTA-519 tumor xenografts after treatment with BAY 1895344 at different doses and dosing schedules. pATR levels decreased remarkably 24 to 48 hours after a single or repeated treatment with 50 mg/kg BAY 1895344, demonstrating direct ATR kinase inhibition (Fig. 3A). γH2AX levels clearly increased 3 to 48 hours after a single or repeated dose.
Figure 4.
Antitumor efficacy of BAY 1895344 in combination with chemotherapy or EBRT. A, Isobologram for the in vitro combination effect of BAY 1895344 and cisplatin on the proliferation of human HT-29 colorectal cancer cells \( (n = 3) \). B, Growth curves of IGROV-1 human ovarian tumors in female nude (nu/nu) mice \( (n = 10/\text{group}) \) treated with BAY 1895344 (10 or 20 mg/kg, once daily, 2 days on/5 days off, per os/orally; 50 mg/kg, twice daily, 3 days on/4 days off, per os/orally) in combination with carboplatin (50 mg/kg, once weekly, intraperitoneally). C, Weights of the IGROV-1 tumors described in B at study end. D, Survival curves of LOVO colorectal cancer cells after treatment with 3 nmol/L BAY 1895344 and different doses of X-ray radiation determined using the clonogenic assay. E, Growth curves of LOVO human colorectal tumors in female nude mice \( (n = 10/\text{group}) \) treated with BAY 1895344 (20 or 50 mg/kg, twice daily 2 on/5 off, per os/orally) in combination with \( \gamma \)-radiation (EBRT). EBRT (5 Gy) was applied directly on the subcutaneously growing tumors on days 12 and 27 after tumor inoculation. Relative tumor area \( (T/C_{\text{rel.area}}) \) is defined as the percentage of the final tumor area on day of control termination versus the initial tumor area (at the time point of starting treatment) of each individual mouse. Asterisks indicate statistical significance, evaluated by survival analysis using the Cox proportional hazards model and longitudinal data were modeled using second-order polynomial curves with random intercepts and slopes for each subject. \( *, P < 0.05; **, P < 0.01; ***, P < 0.001 \) versus vehicle unless indicated otherwise. EC\(_{50}\), half-maximal effective concentration; Gy, Gray; i.p., intraperitoneally; p.o., per os/orally; QD, once daily; 2 QD, twice daily; QW, once weekly.
treatment with 50 mg/kg BAY 1895344, indicating that ATR kinase inhibition may induce DNA damage by abrogation of DDR signaling (Fig. 3B). Further studies demonstrated that the BAY 1895344-evoked increase in H2AX phosphorylation was time-, dose-, and dosing schedule–dependent (Fig. 3C and D). A single dose (once daily × 1) of 30 mg/kg BAY 1895344 increased the level of γH2AX peaking at 8 to 24 hours (Fig. 3C), whereas a single dose of 10 mg/kg or repeated dosing with 3 mg/kg (twice daily × 2 + once daily × 1) was not sufficient to block ATR and cause DNA damage. In contrast, repeated doses of 10 or 30 mg/kg BAY 1895344 increased and prolonged H2AX phosphorylation compared with a single dose of 30 mg/kg. Increasing treatment cycles with BAY 1895344 led to decreased levels of H2AX phosphorylation (Fig. 3D), highlighting the importance of the time point chosen for the determination of a pharmacodynamic signal (γH2AX) to demonstrate target engagement.

In summary, BAY 1895344 displayed strong activity as monotherapy in different CDX models. Dose-dependent antitumor efficacy in vivo correlated with compound exposure in plasma, the reduction of ATR phosphorylation and the induction of DNA damage, demonstrating the anticipated mechanism of action of ATR inhibition with BAY 1895344.

**BAY 1895344 shows synergistic activity in combination with chemotherapy or EBRT**

To assess the potential synergistic activity of ATR inhibition with different DNA damage–inducing chemotherapeutic agents or the tubulin stabilizer docetaxel, antiproliferation-based in vitro combination studies were performed in cancer cell lines of different genetic backgrounds and tumor types. In HT-29 colorectal cancer cells, the interaction of BAY 1895344 with cisplatin was strongly synergistic with a CI of 0.14 (Fig. 4A). Further combinations with cisplatin, bleomycin, or SN-38 were also strongly synergistic (Supplementary Table S4). In contrast, the combination of docetaxel and BAY 1895344 achieved a CI of 1.26, indicating an antagonistic interaction. The CIs for all in vitro combination treatments are summarized in Supplementary Table S4.

The in vivo antitumor efficacy of BAY 1895344 in combination with the chemotherapeutic drug carboplatin was investigated in the IGROV-1 ovarian cancer model. Very low doses of BAY 1895344 (10 or 20 mg/kg, once daily, 20nS/60f = 7% or 14% of MTD) had to be used in combination treatment with carboplatin at MTD to achieve tolerability. BAY 1895344 monotherapy at MTD showed good antitumor efficacy, whereas sub-MTD doses of BAY 1895344 or carboplatin alone showed only weak efficacy. Combination treatment with BAY 1895344 and carboplatin clearly enhanced antitumor efficacy pointing toward a synergistic effect (80% confidence; Fig. 4B and C) at acceptable tolerability (Supplementary Fig. S3A). Although the combination treatments were more effective than respective monotherapies, the efficacy of the tolerated combination treatment did not exceed tumor growth inhibition achieved with BAY 1895344 monotherapy at MTD.

The in vitro radiosensitization potential of BAY 1895344 was assessed in LOVO colorectal cancer cells using the clonogenic assay. Treatment with a noneffective dose of BAY 1895344 in combination with different intensities of γ-radiation clearly reduced the colony-forming ability of LOVO cells compared with radiation alone, confirming the radiosensitization effect of BAY 1895344 (Fig. 4D).

The in vivo antitumor efficacy of BAY 1895344 in combination with EBRT was investigated in LOVO colorectal cancer xenografts. Sub-MTD treatment with BAY 1895344 (20 or 50 mg/kg, twice daily, 20nS/60f = 30% or 60% of MTD) or EBRT alone resulted in weak to moderate antitumor efficacy. Combination treatment with each tested BAY 1895344 dose and EBRT showed strong antitumor efficacy and a synergistic effect (95% confidence, Fig. 4D). Moreover, combination treatment with 50 mg/kg BAY 1895344 and EBRT was more efficacious than radiation alone, as determined on the day of combination group termination. All treatments in the LOVO model were well tolerated with a maximum body weight loss of <10% (Supplementary Fig. S3B).

The rationale for the combination of BAY 1895344 with DNA damage–inducing cancer therapies is supported by the observed synergistic interactions. The observed radiosensitizing effect of ATR inhibition, that however does not have an impact on the safety of EBRT, supports further clinical assessment. However, the tolerability of simultaneous systemic chemotherapy–induced DNA damage and ATR inhibition needs careful evaluation to minimize safety risks.

**BAY 1895344 shows additive to synergistic activity in combination with different DDR inhibitors in vitro and synergy with the PARPi inhibitor olaparib in vivo**

The antiproliferative activity of BAY 1895344 was further assessed in combination with different DDR pathway inhibitors (DDRi) in cancer cell lines of different genetic backgrounds and tumor types. To assess the potential synergistic activity of ATR inhibition together with PARP inhibition, BRCA1–deficient MDA-MB-436 breast cancer cells were treated with BAY 1895344 in combination with the PARPi olaparib, niraparib, rucaparib, and talazoparib. The in vitro interaction of BAY 1895344 with all tested PARPi was strongly synergistic (Fig. 5A–D). Further combinations with DDRi targeting ATM, CHEK1, DNA-PK, and WEE1 demonstrated additive to highly synergistic interactions in ATM- and MRE11A-proficient HT29 and PC-3 cells (CIs summarized in Supplementary Table S4).

The in vivo antitumor efficacy of BAY 1895344 in combination with the PARPi olaparib was investigated using two different CDX models. In the PARPi–sensitive MDA-MB-436 breast cancer model, monotherapy with BAY 1895344 at MTD or sub-MTD (40%; 50 or 20 mg/kg, twice daily, 30nS/40f) or olaparib at MTD (50 mg/kg, once daily) effectively inhibited tumor growth. Combination treatment with BAY 1895344 at sub-MTD and olaparib at MTD showed potent antitumor efficacy, pointing toward a synergistic combination effect (90.5% confidence; Fig. 5E and F). All treatments in the MDA-MB-436 model were well tolerated with a maximum body weight loss of <10% (Supplementary Fig. S3C). In the PARPi–resistant 22Rv1 prostate cancer model, monotherapy with BAY 1895344 at MTD or sub-MTD (40%) resulted in moderate or weak antitumor efficacy. Monotherapy with olaparib at MTD or sub-MTD (40%) failed to inhibit tumor growth, confirming the PARPi resistance of this model. Combination treatment with BAY 1895344 and olaparib at sub-MTDs (40%) delayed tumor growth compared with vehicle and olaparib monotherapy, indicating a synergistic combination effect (95% confidence level; Fig. 5G and H). When looking at final tumor weight, the combination treatment showed improved efficacy also compared with the respective BAY 1895344 monotherapy at sub-MTD (Fig. 5H).

However, the efficacy achieved by BAY 1895344 monotherapy at MTD was comparable with the efficacy achieved by combination therapy in the PARPi–resistant tumor model 22Rv1. All treatments were well tolerated with a maximum body weight loss of <10% (Supplementary Fig. S3D).

These results support the rationale for the combination of BAY 1895344 treatment with DNA repair–compromising cancer therapies. However, the tolerability of simultaneous systemic inhibition of
Antitumor efficacy of BAY 1895344 in combination with PARPi. Isobolograms for the in vitro combination effect of BAY 1895344 and the PARPi olaparib (A), niraparib (B), rucaparib (C), and talazoparib (D) on the proliferation of human MDA-MB-436 breast cancer cells (n = 3).

E, Growth curves of MDA-MB-436 human breast cancer tumors in female NOD/SCID mice (n = 10/group) treated with BAY 1895344 (20 or 50 mg/kg, twice daily, 3 days on/4 days off, per os/orally) in combination with olaparib (50 mg/kg, intraperitoneally, once daily).

F, Weights of the MDA-MB-436 tumors described in E at study end.

G, Growth curves of 22Rv1 human prostate tumors in male SCID mice (n = 10/group) treated with BAY 1895344 (20 or 50 mg/kg, twice daily, 3 days on/4 days off, per os/orally) in combination with olaparib (20 or 50 mg/kg, once daily, intraperitoneally).

H, Weights of the 22Rv1 tumors described in G at study end. Relative tumor area (T/Crel.area) is defined as the percentage of the final tumor area on day of control termination versus the initial tumor area (at the timepoint of starting treatment) of each individual mouse. Asterisks indicate statistical significance, evaluated by survival analysis using the Cox proportional hazards model and longitudinal data were modeled using second-order polynomial curves with random intercepts and slopes for each subject. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus vehicle unless indicated otherwise. EC50, half-maximal effective concentration; i.p., intraperitoneally; p.o., per os/orally; QD, once daily; 2 QD, twice daily.
parallel DDR pathways needs careful evaluation of dose and treatment schedule to ensure safety.

**BAY 1895344 shows improved antitumor activity in combination with the AR antagonist darolutamide in vitro and in vivo**

In LAPC-4 prostate cancer cells, the *in vitro* interaction of BAY 1895344 with the AR antagonist darolutamide achieved a CI of 0.82, indicating an additive interaction (Fig. 6A). Furthermore, combination treatment with BAY 1895344 and darolutamide led to more pronounced downregulation of several genes involved in DNA repair, including *BRCA1*, *EXO1*, and *MCM10*, in comparison with respective monotherapies (Supplementary Fig. S4).

In the hormone-dependent LAPC-4 CDX model, BAY 1895344 monotherapy at sub-MTD (40%) showed a weak to moderate antitumor effect, whereas darolutamide monotherapy exhibited good antitumor efficacy. Combination treatment with BAY 1895344 and darolutamide resulted in enhanced antitumor efficacy, showing significant improvement compared with darolutamide monotherapy on the day of combination group termination (Fig. 6B). All treatments were well tolerated with a maximum body weight loss of <10% (Supplementary Fig. S3E). The triple combination of BAY 1895344, darolutamide, and EBRT resulted in further enhanced antitumor efficacy compared with respective monotherapies and all dual combination treatments and was even more effective than castration (Fig. 6C).

These results support the rationale for the combination treatment of BAY 1895344 with antiandrogen therapy and the addition of EBRT may even further enhance the efficacy of this combination.

**Discussion**

Cancer cells rely on DDR pathways to survive genomic instability in particular by activating ATR kinase (38). In this study, we have shown that BAY 1895344 is a selective low-nanomolar inhibitor of ATR kinase activity, which potently inhibits the proliferation of a broad spectrum of human tumor cell lines of different origins harboring DDR defects, including ATM aberrations. We have demonstrated strong *in vivo* antitumor efficacy of BAY 1895344 in a variety of xenograft models of different tumor indications carrying defects in DNA repair, thus validating the concept of synthetic lethality of tumor-intrinsic DNA-repair deficiencies and ATR blockade.

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**Figure 6.**

Antitumor efficacy of BAY 1895344 in combination with antiandrogen therapy. A, Isobologram for the *in vitro* combination effect of BAY 1895344 and darolutamide on the proliferation of human LAPC-4 prostate cancer cells (*n* = 3). B, Growth curves of hormone-dependent LAPC-4 human prostate tumors in male SCID mice (*n* = 11/group) treated with BAY 1895344 (20 mg/kg, twice daily 3on/4off, per os/orally) in combination with darolutamide (100 mg/kg, once daily, per os/orally). C, Growth curves of LAPC-4 tumors in male SCID mice (*n* = 10/group) treated with BAY 1895344 (20 mg/kg, twice daily 3on/4off, per os/orally) in combination with darolutamide (100 mg/kg, once daily, per os/orally) and EBRT (5 Gy, every 7 days twice). Relative tumor area (T/C_rel.area) is defined as the percentage of the final tumor area on day of control termination versus the initial tumor area (at the time point of starting treatment) of each individual mouse. Asterisks indicate statistical significance, evaluated by survival analysis using the Cox proportional hazards model and longitudinal data were modeled using second-order polynomial curves with random intercepts and slopes for each subject. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 versus vehicle or as indicated. EC_{50}, half-maximal effective concentration; Gy, Gray; p.o., per os/orally; QD, once daily; 2 QD, twice daily; Q7D2, every 7 days twice.
However, further preclinical and clinical investigations are required to understand which DDR defects have the strongest impact on the sensitivity to ATR inhibition. BAY 1895344 demonstrated superiority compared with the ATRis AZD6738 and M6620 (6, 29, 39). Its high potency and selectivity resulted in stronger antitumor activity as monotherapy in a variety of tumor types with DDR defects. We could demonstrate that the plasma exposure of BAY 1895344 clearly covered the antiproliferative concentration levels (IC$_{50}$) of tumor cells. In contrast, the plasma exposure of AZD6738 and M6620 did not exceed the concentrations needed for antiproliferative activity in tumor cells, which is in line with their weak in vivo antitumor efficacy in monotherapy (Fig. 1C and E).

In addition to its potential as monotherapy, BAY 1895344 is likely to be particularly efficacious in combination treatment with DNA damage–inducing or DNA repair–compromising therapies, as indicated in earlier studies (40–44). Here, we have demonstrated in preclinical tumor models that BAY 1895344 exhibits synergistic antitumor efficacy with EBRT in colorectal cancer, with the PARPi olaparib in PARPi-sensitive, HR-defective (BRCA1-mutant) breast cancer as well as in PARPi-resistant prostate cancer and enhanced antitumor activity in combination with the AR antagonist darolutamide in hormone-dependent prostate cancer. The combination of BAY 1895344 with the chemotherapeutic drug carboplatin was associated with dose-dependent toxicity. This restricted overall antitumor efficacy and limited the potential therapeutic value of this combination, in particular because BAY 1895344 monotherapy applied at the MTD was more efficacious than the combination therapy with carboplatin.

The efficacy of EBRT in cancer therapy is mainly due to the ability of ionizing radiation to produce DSBs leading to cell death in tumor tissue. However, the use of EBRT is limited by the sensitivity of normal healthy tissue to radiation, which can cause acute and chronic toxicities. As ionizing radiation also causes an extensive amount of less cytotoxic DNA lesions, such as DNA single-strand breaks (SSB; ref. 45), recent research has focused on the radiosensitization of tumors by combining radiation with DDR-targeted agents, and these studies have demonstrated promising preclinical efficacy (25, 46–49). In this study, BAY 1895344 and EBRT combination treatment of LOVO colorectal cancer xenografts, which have several defects in DDR and mismatch repair (Supplementary Table S3), increased the efficacy of EBRT without affecting tolerability or increasing toxicity. This mechanism-based potential of combining EBRT-induced DNA damage and blockade of ATR-mediated DNA repair with BAY1895344 suggests a powerful new treatment option for patients resistant to radiation, and a possibility to widen the therapeutic window of radiotherapy.

PARPis prevent the repair of SSBs by trapping inactivated PARP onto SS-DNA, resulting in the generation of DSBs during DNA replication, thus activating DNA-repair processes mediated by HR. In tumors with a homologous recombination deficiency (HRD), for example, a BRCA1/2 mutation, PARP-inhibition-induced accumulation of replication errors and DSBs leads to increased genetic instability, and ultimately, cell death (50). The first approval of a PARPi, olaparib, was granted by the FDA in 2014 for use in patients with ovarian cancer with deleterious/suspected deleterious germline BRCA1/2 mutations. However, the efficacy of PARPis is limited by drug resistance (51–53). Not all BRCA1/2 mutation carriers respond to PARP inhibition, and even responders often relapse. Different mechanisms potentially causing PARPi resistance have been described (51, 54, 55). Previous studies have demonstrated that ATR inhibition may be effective in treating cancers with HRD (18). However, ATR is also involved in HR-independent repair pathways indicating the potential of ATRis to improve the antitumor activity of PARPi. PARP and ATR bind to DNA SSBs and initiate distinct DNA-repair mechanisms to inhibit cytotoxic DNA DSB formation. PARP inhibition blocks the DNA-repair complex formation via base-excision repair and induces PARP trapping, which leads to cytotoxic PARP–DNA complexes resulting in DNA breaks. ATR inhibition affects the activation of different DNA-repair pathways, including HR, as part of the RSR. Combined inhibition of ATR and PARP is expected to be highly synergistic by blocking central, but independent, DNA-repair pathways. Therefore, combined inhibition of ATR and PARP could help patients who have relapsed on PARPi therapy by overcoming PARPi resistance but could also enhance antitumor activity in PARPi-sensitive patients. Overall, combined inhibition of ATR and PARP represents a valuable strategy for the improvement of PARPi therapy (56). Here, combined inhibition of ATR with BAY 1895344 and PARP with olaparib resulted in significant reduction of tumor size in PARPi-sensitive, HR-deficient MDA-MB-436 breast cancer xenografts, and in PARPi-resistant 22Rv1 prostate cancer xenografts. Parallel inhibition of different DDR pathways by combining a PARPi, such as olaparib, with the ATR kinase inhibitor BAY 1895344, thus presents novel treatment opportunities in patients with cancer with HRDs sensitive or resistant to PARPi therapy. In fact, a combination of an ATRi with a PARPi is currently under clinical investigation (NCT03462342). Other DDRi combinations may also have clinical impact, as indicated in our in vitro combination studies where the interaction of BAY 1895344 with DNA-PK, CHK1/2, and WEE1 inhibitors was strongly synergistic. However, well-tolerated and efficacious combination treatment regimens need to be established first (57, 58).

AR signaling is a key regulator of the DDR in prostate cancer (21, 24). In recent studies, androgen depletion has been shown to result in the downregulation of DDR genes, impairing DNA repair and increasing sensitivity to radiation (24). AR activity has also been reported to be induced by DNA damage, promoting the resolution of DSBs (59). Thus, AR inhibition can contribute to an HRD phenotype, resulting in sensitivity to DDR inhibition. For example, the AR antagonist enzalutamide in combination with the PARPi olaparib downregulated the expression of DDR genes, resulting in reduced HR efficiency in AR-positive prostate cancer cells (22). Furthermore, combination treatment of enzalutamide with the Chk1/2 inhibitor AZD7762 showed synergistic activity in the inhibition of AR–CDK6–ATR–Chk1 signaling, induction of ATM phosphorylation, and apoptosis in hormone-dependent prostate cancer cells (21). Here, we investigated for the first time the combination of ATR inhibition and antiandrogen therapy. Combined treatment with BAY 1895344 and darolutamide, a novel nonsteroidal AR antagonist (60) that has successfully completed a clinical phase III trial in the indication of nonmetastatic prostate cancer (61), demonstrated significantly reduced expression of DDR genes in prostate cancer cells in vitro and significantly improved antitumor efficacy in the hormone-dependent LAPC-4 prostate cancer CDX model in vivo. Adding EBRT to the combination treatment even further enhanced the therapeutic efficacy. Therefore, induction of DNA damage by radiation and reduced DNA-repair capability by the AR antagonist darolutamide combined with the ATR inhibitor BAY 1895344 could present a new treatment option for patients with hormone-dependent prostate cancer.

In summary, BAY 1895344 is a novel ATR kinase inhibitor that exhibits strong monotherapy efficacy in cancers with DDR deficiencies of different tumor histologies, as well as synergistic activity in combination with DNA damage–inducing or DNA repair–compromising therapies. This may provide new treatment options and improve the therapeutic efficacy of standard-of-care therapies. BAY 1895344 is
Discursively under clinical investigation in patients with advanced solid tumors and lymphomas (NCT03188965).

Disclosures of Potential Conflicts of Interest

All authors are employed at and have ownership interest (including patents) in Bayer AG.

Authors’ Contributions


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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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