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**ATAXIA TELANGIECTASIA AND RAD3 RELATED KINASE DRIVES
BOTH THE EARLY AND THE LATE DNA DAMAGE RESPONSE
TO THE MONOFUNCTIONAL ANTITUMOR ALKYLATOR S23906.**

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Running Title: S23906 is a potent inducer of replication stress

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SYNOPSIS

Numerous anticancer agents and environmental mutagens target DNA. Although all such compounds interfere with the progression of the replication fork and inhibit DNA synthesis, there are marked differences in the DNA damage response pathways they trigger, and the relative impact of the proximal or the distal signal transducers on cell survival is mainly lesion-specific. Accordingly, checkpoint kinase inhibitors in current clinical development show synergistic activity with some DNA-targeting agents, but not with others. Here, we characterize the DNA damage response to the antitumor acronycine derivative S23906 that forms monofunctional adducts with guanine residues in the minor groove of DNA. S23906 exposure is accompanied by specific recruitment of RPA at replication sites and rapid Chk1 activation. In contrast, neither MRN (Mre11-Rad50-Nbs1) nor ATM (ataxia-telangiectasia mutated), contributes to the initial response to S23906. Interestingly, genetic attenuation of ATR (ATM and RAD3-related) activity inhibits not only the early phosphorylation of histone H2AX and Chk1, but interferes also with the late phosphorylation of Chk2. Moreover, loss of ATR function or pharmacological inhibition of the checkpoint kinases by AZD7762 is accompanied by abrogation of the S-phase arrest and increased sensitivity towards S23906. These findings identify ATR as a central coordinator of the DNA damage response to S23906 and provide a mechanistic rationale for combinations of S23906 and similar agents with checkpoint abrogators.

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INTRODUCTION

Eukaryotic cells have developed numerous genome maintenance processes in order to coordinate DNA repair and cell cycle progression. The major regulators of the DNA damage response are the two phosphatidylinositol 3-kinase-like kinases (PIKKs), ataxia-telangiectasia mutated (ATM) and ATM and RAD3-related (ATR) [1,2]. ATM initiates the cellular response to DNA double strand breaks (DSBs) and is recruited to damaged sites by the Mre11-Rad50-Nbs1 (MRN) complex via its interaction with the C-terminal domain of Nbs1 [3,4]. ATM, which exists as an inactive dimer, is activated through autophosphorylation of the Ser1981 residue leading to formation of catalytically active monomers [3]. In comparison, the primary function of ATR is to monitor DNA replication [5]. ATR regulates the firing of replication origins and the repair of damaged replication forks, and is activated to prevent the premature onset of mitosis [6,7]. ATR is recruited by the ATR-interacting protein (ATRIP) to regions of replication protein A (RPA)-coated stretches of single-stranded DNA (ssDNA) that are generated by decoupling of helicase and polymerase activities at stalled replication forks [8-11]. Direct binding of ATRIP to RPA-coated ssDNA is required for stable recruitment of the ATR-ATRIP complex to damaged sites [10,12]. Full activation of ATR requires TopBP1 (topoisomerase-binding protein-1) that is recruited to the stalled replication fork through its interaction with the 9-1-1 complex [13-17].

ATM and ATR have both common phosphorylation substrates, like γ -H2AX, and distinct substrates. For example, ATM phosphorylates and activates the distal transducer kinase, Chk2, while ATR preferentially phosphorylates the distal Chk1 kinase [18,19]. There is substantial evidence for cross-talk between the two pathways, and activation of one pathway is usually followed by activation of the other, although the sequence of events appears to be agent-specific. For example, ATR is activated in response to DNA lesions induced by camptothecin and UV or by replication stress in the presence of hydroxyurea (HU) followed later by ATM activation [20,21]. In the case of camptothecin, the ATR-dependent ATM activation is rapid, leading to strong ATM activation after 1 hour drug exposure [22]. Conversely, ATR activation following ionizing radiation (IR) is a secondary process which depends on both ATM and MRN [23,24]. A strong DNA damage response may require several PIKKs. For example, camptothecin-induced phosphorylation of RPA32 is fast and depends on both ATR and DNA-PK [25,26]. Similarly, RPA32 phosphorylation following UV or HU exposure, which is a slower process that probably occurs after replication fork collapse, requires both ATR, ATM and MRN [27-30].

A precise understanding of the individual steps of the DNA damage response is of clinical importance since a broad spectrum of cancer chemotherapeutic agents are targeting DNA. Most of the primary lesions induced by these drugs are later processed into highly toxic DSBs in a replication-dependent manner [20,31-33]. Cancer cells with certain genetic aberrations, like BRCA2 deficiency, can be targeted by agents that interfere with DNA repair pathways [34]. Interference with the DNA damage response could also be an attractive strategy. Until recently, attempts to translate such strategies into the clinic were limited by high general toxicity and lack of specificity of available checkpoint abrogators [35]. However, new checkpoint kinase inhibitors are currently in clinical development [36]. AZD7762, a selective Chk1/2 inhibitor, has been shown to markedly increase the sensitivity of cells to gemcitabine, a nucleoside analogue, by converting the gemcitabine-related stalled

replication forks into DSBs [37,38]. Another checkpoint abrogator, PF-0477736, which preferentially inhibits Chk1, also potentiates the cytotoxic activity of gemcitabine and has shown clinical activity [39,40]. In addition to nucleoside analogues, this strategy might be applicable to other drugs which interferes with the replication fork [37,39]. A potential complication with this approach is that the involvement of the different pathways seems to be lesion-specific [20,41-43].

The variability is particularly striking for alkylating agents, a widely used class of antitumor compounds. Indeed, although all DNA adducts can interfere with the progress of the replication fork and inhibit DNA synthesis, there are marked differences in the DNA damage response pathways they trigger. For example, if we consider the bifunctional alkylating agents, ATR is needed for the DNA damage response to cisplatin and BCNU (1,3-Bis(2-chloroethyl)-1-nitrosourea), but not to oxaliplatin or carboplatin [41-43]. Moreover, while ATR plays an important role in cisplatin survival, Chk1, its downstream target, does not [43]. The DNA damage response is equally complex with regard to monofunctional alkylating agents. The early response to hedamycin does not depend on either ATR, ATM or DNA-PK [44]. In contrast, adozelesin activates the ATR/Chk1 pathway, while iriflufen specifically induces the ATM/Chk2 signaling in replicating cells [45-47]. Therefore, deciphering the precise steps involved in the DNA damage response to individual agents will be necessary to rationalize new therapeutic schedules.

In this study, we characterize the DNA damage response to S23906, an anticancer acronycine derivative with novel structural features. S23906 forms bulky monofunctional adducts with the exocyclic amino group of guanine residues in the minor groove of DNA leading to local helix opening [48,49]. Incomplete repair of the primary S23906-adducts by the nucleotide excision repair pathway leads to formation of secondary DNA lesions in form of DSBs when the replication fork collides with the adducts [32,50]. Interestingly, S23906 has been described as a strong inhibitor of DNA synthesis in comparison with other alkylating agents [51], and is also a rapid inducer of γ -H2AX foci [52]. Our results show that the initial formation of γ -H2AX foci following S23906 exposure is strongly correlated with sites of DNA synthesis within individual cells. Interestingly, genetic or pharmacological attenuation of ATR activity inhibits not only the early phosphorylation of histone H2AX and Chk1, but also interferes with the late phosphorylation of Chk2. Moreover, loss of ATR function is accompanied by the abrogation of S-phase arrest and increased sensitivity towards S23906. These data identify ATR as a central coordinator of both the early and the late DNA damage response to S23906 and provide a mechanistic rationale for combinations of S23906 and similar agents with checkpoint abrogators.

MATERIALS AND METHODS

Chemicals

The acronycine derivative S23906 (cis-1,2-diacetoxy-3,14-dihydro-3,3,14-trimethyl-6-methoxy-7H-benz(b)pyrano(3,2-d)acridin-7-one) was obtained from Institut de Recherches Servier (Croissy sur Seine, France) while SN-38 (7-ethyl-10-hydroxycamptothecin), the active metabolite of camptothecin was purchased from Abatara technology (Xi'an, China). Doxycycline and UCN-01 were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). AZD7762 was purchased from Axon Medchem (Groningen, the Netherlands). Protease inhibitors were obtained from Roche Diagnostics (Mannheim, Germany).

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's Medium (InVitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (Perbio Science, Brebières, France). Wild type donor (GM03657), ATR-Seckel donor (GM18367, ATR "low") and ataxia telangiectasia (A-T) donor lymphoblastoid cells (GM03189, ATM deficient) were grown in RPMI-1640 medium (InVitrogen) supplemented with 5% fetal calf serum (Perbio Science, Brebières, France). We defined GM18367 cell line as ATR "low" because it is derived from a Seckel syndrome patient with a splicing mutation (2101A→G) in the ATR gene allowing little residual expression of ATR [53]. ATRkd-expressing cells were constructed from GM847 SV40-transformed fibroblasts by transfection with cDNA encoding kinase-inactive ATR under the control of a doxycycline-responsive promoter [54] and were maintained in MEM (InVitrogen) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine (Eurobio, Les Ulis, France). For overexpression of kinase-inactive ATR, ATRkd-expressing cells were incubated with 5 µg/mL doxycycline for 48 hours prior to the experiments. Lymphoblasts and ATRkd-expressing cells were kindly provided by Filippo Rosselli (Institut Gustave-Roussy, Villejuif, France). All media were supplemented with 100 units/mL penicillin, 100 µg/ml streptomycin (PanPharma, Fougères, France) and 2,5 µg/mL plasmocin (Lonza, Levallois-Perret, France).

Antibodies

The P-Thr68-Chk2 (# 2661), Chk2 (clone 1C12, # 3440), P-Ser317-Chk1 (# 2344), P-Ser345-Chk1 (# 2341), Chk1 (clone 2G1D5, # 2360), P-Ser1981-ATM (clone 10H11.E12, # 4526), ATM (clone D2E2, # 2873), RPA32 (clone 4E4, # 2208), and the RPA70 (# 2267) -directed antibodies were purchased from Cell Signaling Technology (Ozyme, Saint Quentin en Yvelines, France). The TopBP1 (# 611874) and the β-actin-directed antibodies (clone AC-15, # A-54421) were purchased from BD Biosciences (Le Pont de Claix, France) and Sigma-Aldrich, respectively. The H2AX (# 07-627) and γ-H2AX (# 05-636) -directed antibodies were purchased from Millipore (Lake Placid, NY). The Mre11 (clone 12D7), rad50 (clone 13B3) and Nbs1 -directed antibodies were purchased from Novus Biologicals (Interchim, Montluçon, France). Finally, the Lamin B (clone M-20, # sc-6217), ATR (clone E-14, # sc-23736) and Cdc25A (clone F6, # sc-7389) -directed antibodies were purchased from Santa Cruz Biotechnology (Tebu-Bio, Le Perray en Yvelines, France). HRP and fluorescent dye-conjugated antibodies were purchased from Jackson Immunoresearch (Bar Harbor, ME).

Cell Cycle Analysis

For cell cycle analysis, experiments were carried out as described previously [55]. Briefly, HeLa cells were exposed to 1 µM of S23906 for 1 hour followed by 6 hours in drug-free media. When indicated, cells were co-incubated with 100 nM of UCN-01 and 1 µM of S23906 for 1 hour and post-incubated for 6 hours in presence of 100 nM UCN-01. ATRkd-expressing cells were incubated in the absence or presence of 5 µg/mL doxycycline for 48 hours prior to cell cycle analysis. ATRkd cells were then exposed to 1 µM of S23906 for 1 hour followed by 12 hours post-incubation in drug-free medium. Cell cycle experiments were conducted with a final concentration of S23906 equal to 1 µM to show that cell cycle modification occurs at concentrations close to the IC50 values of S23906 as determined by the MTT assay. Cells were washed and fixed with 70% ethanol. Cells were resuspended in PBS and incubated for 30 minutes at room temperature in a solution containing 5 µg/mL DAPI (Sigma-

Aldrich) and 100 µg/mL RNase (Sigma-Aldrich). Samples were analyzed with a LSR II flow cytometer and DIVA software (BD Biosciences, France).

Immunofluorescence and microscopy

Cells were incubated with different concentrations of S23906 at 37°C for the indicated times. For short exposure time (1 hour), 5 µM S23906 was used because it gave a strong and very reproducible DNA damage signal by 1 hour in both HeLa, lymphoblastoid or ATRkd cells. When cells were post-incubated for up to 12 hours (Figures 5A and 5C), the concentration used was decreased to 2 µM S23906 to avoid unspecific labeling due to the induction of cell death [52]. Immunofluorescence experiments were carried out as described previously [56]. When indicated, coverslips were washed twice in PBS and resuspended in ice-cold CSK-lysis buffer (150 mM NaCl, 3 mM MgCl₂, 1% Triton X100, 50 mM HEPES pH 7.4, 30 mM sucrose and protease inhibitors) and kept at 4°C for 5 minutes before fixation in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, U.S.A). The antigens were revealed by using the indicated primary antibodies. DNA was counterstained by DAPI. Replicating cells were labeled by incorporation of EdU (5-ethynyl-2'-deoxyuridine) (Click-iT™ EdU Alexa Fluor® 488 Imaging Kit, # C10337, Invitrogen, Cergy-Pontoise, France) for 30 minutes prior to drug addition. EdU labeling was carried out according to the manufacturer's instructions. Images were collected using a BX61 microscope and cell F imaging software (Olympus). Fluorescence intensities were measured by MetaMorph software (Universal Imaging Corporation, Downingtown, PA). The background over noncellular regions was subtracted. At least 100 cells were analyzed per sample. Values represent the averages of at least two independent experiments.

Immunoblotting

Cells were incubated with different concentrations of S23906 at 37°C for the indicated times, washed in PBS, counted and lysed for 30 minutes at 4°C in SDS-polyacrylamide gel loading buffer. Alternatively, cells were washed twice in PBS and resuspended in ice-cold CSK-lysis buffer (150 mM NaCl, 3 mM MgCl₂, 1% Triton X100, 50 mM HEPES pH 7.4, 30 mM sucrose and protease inhibitors) and kept at 4°C for 5 minutes. Soluble proteins were separated from detergent-resistant proteins by centrifugation at 1500 g for 5 min. For short exposure time (1 hour), 5 µM S23906 was used because it gave a strong and very reproducible DNA damage signal by 1 hour for all cell lines tested. When cells were post-incubated for up to 24 hours (Figures 3G and 5B), the concentration used for S23906 was decreased to 2 µM to avoid unspecific labeling due to the induction of cell death [52]. Proteins were resolved on a linear-gradient SDS-polyacrylamide gel (5-15%) and blotted onto nitrocellulose membranes (Biorad). Membranes were saturated by TBST-milk (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Tween 20 and 5% dehydrated skimmed milk) and the antigens were revealed by immunolabeling. Antigens were detected using an enhanced chemiluminescence kit (Amershan Biosciences).

Depletion of ATR or ATM by small interfering RNA

Small interfering RNA (siRNA) transfections were performed accordingly to the manufacturers' instructions with HiPerfect transfection reagent (Qiagen, Courtaboeuf, France) and 10 nmol/L final concentrations of oligos. siRNA duplexes of 25-nucleotide length were purchased from Invitrogen. The siRNA target sequence of ATR is 5'-CAUCAGGAGUUGCUUUCUAUUUUAU-3'. The siRNA target sequence

of ATM is 5'- UAAGCAGAUCAUUAAGUACUAGACU-3'. Scrambled siRNA (Stealth RNAi™ siRNA Negative Control LO GC) were purchased from InVitrogen. Transfection mixtures were removed after 6 hours and cells were incubated with complete medium for 48 hours before treatment with S23906. Cells were then processed for either immunofluorescence or immunoblotting as described above.

Viability Assays

The growth inhibitory activity of S23906 was determined by the MTT (methylthiazolyldiphenyl-tetrazolium bromide) viability assay, as previously described [57]. All values are averages of at least three independent experiments, each done in duplicate. ATRkd-expressing cells were incubated in the absence or presence of 5 µg/mL doxycycline for 48 hours prior to the start of the viability assays. ATRkd-expressing cells were then exposed to S23906 for 1 hour followed by 5 generation doubling times in drug-free medium in the absence or presence of doxycycline. Alternatively, the ATRkd-overexpressing fibroblasts were exposed to S23906 for 1 hour in the absence or presence of 50 nM AZD7762. Then the cells were washed and incubated for 5 generation doubling times in absence or presence of 50 nM AZD7762.

Single cell gel electrophoresis (comet assay)

Lymphoblasts were exposed to the indicated S23906 concentrations at 37°C in the dark and subjected to single cell electrophoresis under alkaline conditions as described [33]. Image analysis was performed by using Komet 5.5 software (Kinetic Imaging, Nottingham, UK). At least 100 cells were analyzed per condition.

RESULTS

S23906 specifically induces early DNA damage response in replicating cells

To establish the relationship between early γ -H2AX induction and the replication status, HeLa cells were labeled with EdU, a thymidine analogue, prior to treatment with S23906. While no γ -H2AX signal could be observed in the absence of drug, S23906 exposure was accompanied by an important increase in γ -H2AX labeling. Importantly, the majority of cells that were positive for γ -H2AX were also positive for EdU, whereas no detectable γ -H2AX staining was observed for non-replicating cells even at drug concentration as high as 5 µM (Figure 1A and Supplementary Figure S1). Further analysis revealed that the S23906-induced γ -H2AX foci were associated with sites of DNA synthesis within individual cells (Figure 1B), suggesting that the early induction of the DNA damage response is limited to actively replicating DNA regions.

S23906 exposure was accompanied by rapid modifications of the cell cycle distribution. Indeed, 1 hour exposure to S23906 (1 µM) followed by 6 hours post-incubation in drug-free media was accompanied by a marked enrichment of the S-phase fraction (Figure 1C, compare the 1st and 3rd column), thus demonstrating that, at relevant cytotoxic concentrations, S23906 induces a rapid and strong S-phase slow-down. This effect was completely abolished by UCN-01, an inhibitor of the Chk1 and Chk2 checkpoint kinases (Figure 1C, compare the 3rd and the 4th panels). Interestingly, the cell cycle distribution of S23906-treated cells was modified at UCN-01 concentrations as low as 100 nM. This suggests that it is principally Chk1 which is involved in the initial cell cycle response, since Chk2 inhibition requires higher concentrations of UCN-01 [58]. Together, these results demonstrate that S23906

interferes with the replication fork and induces a rapid DNA damage response as visualized by histone H2AX phosphorylation and accumulation of cells in the S phase of the cell cycle.

ATM does not participate in the early DNA damage response to S23906

To identify the key factors needed for the early DNA damage response to S23906, we first determined the activity of ATM. Immunofluorescence microscopy was used to determine the activation of ATM, as measured by ATM autophosphorylation of Ser1981 after 1 hour exposure to 5 μ M S23906 or to 200 nM SN-38 (Figure 2A). The results show that S23906 does not modify the phosphorylation status of ATM, compared to untreated control cells, whereas SN-38 was a strong activator of ATM (Figure 2A). Interestingly, S23906 did not interfere with the physiological autophosphorylation of ATM during mitosis [59], since the ATM Ser1981 phosphorylation of mitotic cells was not influenced by S23906 exposure (Figure 2A, arrows and Supplementary Figure S2). In agreement with the results for ATM, S23906 had only marginal influence on the phosphorylation of Chk2 on Thr68 (Figure 2B, compare the 1st and the 2nd column), in comparison with SN-38 exposed cells (Figure 2B, 3rd column). Thus, even at high drug concentration, no activation of the ATM pathway can be detected after 1 hour S23906 exposure.

Because phosphospecific antibodies can sometimes cross-react, we confirmed the immunofluorescence data by immunoblotting. Again, our results show that S23906 poorly activates ATM or Chk2, in comparison with SN-38 (Figure 2C). Furthermore, S23906 exposure was not accompanied by the recruitment of Rad50 and Mre11 to the insoluble fraction of CSK-buffer extracted cells, in contrast to what was observed for cells exposed to SN-38 (Figure 2D) or reported for UV irradiation [60]. These data were further confirmed by the absence of Nbs1 focalization in S23906-exposed cells (Figure 2E). Together, these results strongly suggest that the early DNA damage response to S23906 is not mediated via ATM or MRN.

S23906 induces recruitment of RPA to actively replicating DNA regions and activates the ATR/Chk1 pathway

Signaling of replication stress is initiated by regions of RPA-coated single-stranded DNA (ssDNA) that are generated by decoupling of helicase and polymerase activities at stalled replication forks. Short (1 hour) S23906 exposure was accompanied by strong chromatin recruitment of RPA70/RPA1, one the three RPA subunits (Figure 3A), which was selective for replicating cells (Figure 3A and Supplementary Figure S3). The recruitment was not restricted to RPA70 but was equally observed for another RPA subunit, RPA32/RPA2 (Figure 3B). The S23906-induced RPA foci were strongly associated with actively replicating DNA regions (Figure 3B), suggesting that S23906, in addition to its capacity to arrest nucleotide incorporation, is able to uncouple the helicase and polymerase activities. Interestingly, the formation of RPA foci following S23906 exposure was not accompanied by hyperphosphorylation of RPA32, in contrast to what was observed for SN-38 (Figure 3C), suggesting that replication fork collapse occurs later for S23906-treated cells than for SN-38 treated cells.

RPA-coated single-stranded DNA (ssDNA) is needed for recruitment of the ATR-ATRIP complex to damaged sites [8-11] whereas subsequent recruitment of TopBP1 is needed for optimal activation of ATR [13-15,17]. Short S23906 exposure was accompanied by recruitment of TopBP1 to the RPA-coated regions as shown by immunocytochemistry (Figure 3D, arrows). This was accompanied by rapid

activation of ATR, as indicated by the formation of phosphorylated Chk1 on both Ser317 and Ser345 residues (Figure 3, E and F) which was selective for actively replicating cells (Supplementary Figures S4 and S5). Activation of Chk1 was accompanied by a rapid and sustained loss of Cdc25A, a downstream Chk1 target, thus limiting the capacity of the cells to undergo S-phase progression (Figure 3G). These results suggest that ATR and its downstream targets are rapidly activated in response to S23906 exposure.

The early DNA damage response to S23906 is ATR-dependent

For further characterization of the ATR-mediated early DNA damage response to S23906, we used three different genetic models. First, the capacity of S23906 to induce γ -H2AX foci and Chk1 phosphorylation was determined for three patient-derived lymphoblastoid cell lines: GM03657 (normal donor), GM18367 (ATR-Seckel donor, ATR "low") and GM03189 (ataxia telangiectasia donor, ATM deficient). S23906 exposure of normal and ATM-deficient cells was accompanied by formation of comparable levels of γ -H2AX while no γ -H2AX staining was observed for the ATR-Seckel cells (Figures 4A and 4B). Accordingly, Chk1 phosphorylation induced in response to S23906 was strongly diminished in ATR-Seckel cells compared to normal and ATM-deficient cells (Figure 4B). Importantly, the lack of γ -H2AX staining and Chk1 phosphorylation in the ATR-Seckel cells was not due to differences in the induction of DNA damage, since 1 hour S23906 exposure resulted in comparable levels of DNA damage for the 3 cell lines as determined by the alkaline comet assay (Figure 4C).

In agreement, overexpression of a kinase-dead form of ATR (ATRkd) in GM847 human fibroblasts (Figure 4D, right panel) drastically decreased the formation of S23906-induced γ -H2AX foci, whereas a partial reduction was observed in response to SN-38 (Figure 4E). In contrast, attenuation of ATR activity had no detectable effect on the initial chromatin recruitment of RPA70 following S23906 exposure (Figure 4F). Therefore, while similar levels of ssDNA are formed in the absence or presence of functional ATR, the subsequent phosphorylation of H2AX is strongly ATR-dependent.

To further confirm the central role of ATR in the early DNA damage response to S23906, RNA interference experiments were carried out in HeLa cells. By 48 hours after siRNA transfection, protein levels of both ATR and ATM were specifically knocked down to less than 20% of control levels (Figure 4G). Importantly, when ATR expression was knocked down, phosphorylation of Chk1 on its Ser317 residue was markedly reduced following S23906 treatment (Figure 4G). In clear contrast, ATM knock down did not influence the ability of the cells to induce Chk1 phosphorylation (Figure 4G). Moreover, ATR knock down, but not ATM knock down, reduced γ -H2AX foci formation following S23906 treatment (Figure 4H). Taken together, our results clearly show that the DNA lesions induced by S23906 quickly activate ATR, but not ATM.

Influence of ATR on the delayed response to S23906

Although S23906 exposure was not accompanied by the formation of phosphorylated histone H2AX in cells with attenuated ATR activity by 1 hour, this was only a transient effect (Figure 5A). Indeed, when these cells were post-incubated in drug-free media, detectable γ -H2AX appeared after 2 hours, and by 6 hours post-incubation, the γ -H2AX signal for cells with attenuated ATR activity became even higher than it was for cells with catalytically active ATR (Figure 5A).

Chk1 was also differentially phosphorylated in cells with active or attenuated ATR activity (Figure 5B), although with a different kinetics than for histone H2AX (Figure 5A). In contrast, ATR activity had no influence on the hyperphosphorylation of RPA32 (Figure 5B). These data suggest that S23906 is a strong inducer of replication stress which leads to rapid ATR activation as indicated by phosphorylation of H2AX and Chk1. Subsequently, the initial lesions are transformed into secondary lesions, such as DSBs, leading to activation of other pathways resulting in additional H2AX phosphorylation as well as phosphorylation of RPA32. Accordingly, the induction of Thr68-phosphorylated Chk2 following S23906 treatment followed a similar kinetics as γ -H2AX (Figures 5A and 5C, open symbols). Intriguingly, attenuation of ATR activity inhibited not only the early phosphorylation of Chk1 and histone H2AX but interfered also with the late phosphorylation of the Thr68 residue on Chk2 (Figures 5B and 5C). This is an important observation since both Chk1 and Chk2 are involved in cell cycle regulation [19].

To determine the impact of the diminished activation of Chk1 and Chk2 on the cell cycle progression, GM847 cells overexpressing or not kinase-dead ATR were exposed to S23906 for 1 hour followed by 12 hours post-incubation in drug-free media and processed for flow cytometry analysis. The results show, that attenuation of ATR activity interferes with the S23906-induced S-phase arrest (Figure 5D, compare the 2nd and 4th panels). Interestingly, attenuation of functional ATR was also accompanied by 2-3 fold increased sensitivity to S23906 (Figure 5E). Taken together, these results indicate that ATR plays a crucial role in both the initial and the delayed response to S23906.

AZD7762, a novel checkpoint abrogator potentiates S23906 activity

Even in cells with attenuated ATR activity, the DNA damage response is eventually activated leading to induction of distal signal transducers like Chk1 and Chk2 (Figures 5B and 5C). Therefore, inhibition of the distal checkpoint kinases rather than the proximal kinases (e.g. ATR, ATM) might be an alternative strategy to improve the cytotoxic activity of S23906 activity. To test this hypothesis, S23906 was combined with the novel checkpoint abrogator AZD7762, a selective inhibitor of Chk1 and Chk2 [37]. As shown in Figure 5F, AZD7762 potentiated the cytotoxic activity of S23906 approximately 5 fold. A similar effect of AZD7762 was observed for HeLa cells (Supplementary Figure S6). Interestingly, the potentiating effect of AZD7762 on S23906 was comparable with the potentiation of AZD7762 for SN-38, a combination which is under clinical evaluation (Supplementary Figure S7).

DISCUSSION

S23906 forms bulky monofunctional DNA adducts which induce local base-unpairing [49]. Unrepaired adducts can be converted into DSBs when they encounter the advancing replication fork [32,50]. By 24 hours, S23906 exposure is associated with strong γ -H2AX formation which is specific for S-phase cells [32], in agreement with observations made for other DNA interacting agents [20,31,33]. In contrast, little is known about the early response to S23906. It is increasingly recognized that the generation of γ -H2AX foci may be associated with DSBs as well as with replication fork stalling. This distinction is important since the early activation of the ATR/Chk1 pathway in response to replication stress controls the stabilization of stalled replication forks, S-phase progression, and the resumption of DNA synthesis [5]. Therefore, the early response to replicative stress is crucial and determines, at least

in part, the subsequent formation of DSBs. A better knowledge of the early events associated with processing of the S23906 adducts should provide a rationale for combinations of S23906 and similar agents with drugs that target the DNA damage response.

Here, we report that the initial formation of γ -H2AX following S23906 exposure is strongly correlated with replication and that the γ -H2AX foci are closely associated with sites of DNA synthesis. This is accompanied by specific recruitment of RPA, suggesting that single-stranded DNA regions are formed at the stalled replication forks, likely due to uncoupling of helicase and polymerase activities. Consistent with the formation of single stranded-DNA, the initial DNA damage response, is strongly, if not exclusively, ATR-dependent. In contrast, MRN and ATM have no detectable influence at early times even at high drug concentration (e.g. 5 μ M).

However, additional pathways must be activated later on, since the γ -H2AX signal increases over time, even in cells with attenuated ATR activity and in the absence of drug. The delayed activation of γ -H2AX could be explained by two different, non-exclusives, models. First, DSBs may arise at later time points, possibly due to DSB formation at sites of collapsed replication forks [61], resulting in activation of ATM and DNA-PK, as has been suggested for UV-induced DNA lesions [21,61]. In agreement with this model, attenuation of ATR activity delayed the activation of both Chk1 and Chk2 kinases following exposure to S23906, but did not completely abrogate them, suggesting that parallel pathways are able to activate the two distal checkpoint kinases.

Alternatively, we might have a step-wise process like the one which has been proposed for camptothecin [20], where stalling of the replication fork activates ATR thereby leading to formation of γ -H2AX. Then, MRN could be recruited through γ -H2AX and cooperate with ATM to subsequently activate Chk2 [20]. In agreement with the stepwise model, we show that attenuation of ATR activity delays Chk2 activation following S23906 exposure. In contrast to what has been reported for camptothecin, UV adducts and iriffulven [21,22,46], the ATM/Chk2 pathway is activated relatively late following S23906 exposure. RPA32 hyperphosphorylation is also a late phenomenon compared to camptothecin and adozelesin [25,26,31], suggesting that an important fraction of the RPA remains associated with the replication fork and is only rerouted to DNA damage processing and repair at later times [62]. Together, these results strongly suggest that, despite common features, both the early DNA damage response and the early processing of the DNA lesions, are lesion-dependent and demonstrate that, even among a unique class of anticancer compounds, the cellular response may vary significantly.

Importantly, the early S-phase arrest observed in S23906-treated cells is strongly dependent on the ATR/Chk1 pathway as shown by both genetic (inducible overexpression of kinase-dead ATR) and pharmacological (low concentrations of UCN-01) pathway modulation. These observations are important since several Chk1 inhibitors are currently undergoing clinical trials in combination with irinotecan and gemcitabine, two DNA interacting agents that induce replication stress [63]. Chk1 inhibition strongly potentiates the cytotoxic activity of some DNA-damaging agents in tumor cells with intrinsic G1/S checkpoint defects [37,39]. Moreover, it has recently been shown that loss of the ATR/Chk1 pathway increases the caspase-3-dependent response to HU-induced replicative stress [64]. These data suggest that combining S23906 with checkpoint abrogators might increase its cytotoxic activity. In agreement, we here show that genetic attenuation of ATR, as well as inhibition of the

checkpoint kinases by AZD7762 or UCN-01, do indeed potentiate the cytotoxicity of S23906.

It is interesting to note that a similar strategy does not apply to cisplatin, a widely used alkylating agent. Indeed, although ATR inhibition does potentiate cisplatin activity, Chk1/2 inhibition does not [42,43]. The discrepancy may be explained by the ability of ATR to phosphorylate not only Chk1 but also additional substrates, among which some are involved in various repair pathways [36]. It is likely that the nature of the lesions generated by cisplatin is more complex than those induced by S23906. Indeed, cisplatin is a bifunctional alkylating agent that can induce interstrand cross-links, DNA-protein cross-links as well as intrastrand cross-links, where each type of DNA lesion may induce a distinct DNA damage response. Thus, a careful characterization of the lesions and their consequences on the DNA damage response they induce needs to be taken into account for the future development of ATR or checkpoint kinase inhibitors. Interestingly, it has recently been demonstrated that inhibition of the Chk1/2 kinases by AZD7762 can also activate a DSB response in gemcitabine-treated cells making cells with deficiencies in DSB repair particularly sensitive to the combination of AZD7762 and gemcitabine [38]. In contrast, one would predict, that cells with strong DSB repair capacity would not be sensitive to such combinations.

Together, these data demonstrate that a precise knowledge of the cellular context as well as a detailed characterization of the cellular events activated by replication fork stalling will be required for efficient combinations of DNA damaging agents and pathway inhibitors in the clinic.

AUTHOR CONTRIBUTION

Daniele G. Soares carried out the immunofluorescence studies, the image analysis and the viability assays. She participated in the cell cycle analysis and the comet assay and helped to draft the manuscript. Aude Battistella carried out the immunoblotting assays and participated in the immunofluorescence studies. Céline J. Rocca participated in the cell cycle analysis and the comet assay. Renata Matuo participated in the immunoblotting assays and the viability assays. João A. P. Henriques participated in the design of the study. Annette K. Larsen conceived the study, and participated in its design and coordination. AKL also helped to draft the manuscript. Alexandre E. Escargueil conceived, designed and coordinated the study. Alexandre E. Escargueil drafted the manuscript.

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FIGURE LEGENDS

Figure 1: S23906 selectively targets replicating cells

(A) Proliferating HeLa cells were incubated with EdU for 30 min followed by 1 hour exposure to S23906 (5 μ M). Cells were fixed and processed for EdU and γ -H2AX staining and the DNA was counterstained by DAPI. (B) Increased magnification of the cells shown in A. The merged image illustrates the colocalization of EdU and γ -H2AX (indicated with arrows). The dotted circle represents a non-replicating cell. (C) Cell cycle distribution of HeLa cells incubated in the absence or presence of S23906 and UCN-01. From left to right, cells were mock treated for 7 hours (1st panel), or exposed to 100 nM UCN-01 for 7 hours (2nd panel), to S23906 (1 μ M) for 1 hour followed by 6 hours post-incubation in drug-free media (3rd panel) or co-incubated with 100 nM UCN-01 and 1 μ M S23906 for 1 hour followed by 6 hours post-incubation in media with 100 nM UCN-01 (4th panel). The values represent the averages of four independent experiments. Standard deviations are indicated by error bars.

Figure 2: S23906 does not activate the ATM pathway in replicating cells

(A) HeLa cells were mock treated or exposed for 1 hour to S23906 (5 μ M) or SN-38 (200 nM). Cells were fixed and processed for immunolabeling with an antibody directed against Ser1981-phosphorylated ATM to determine the levels of ATM autophosphorylation. The fluorescence intensities were quantified by Metamorph analysis and are indicated in arbitrary units (a.u.). Arrows indicate mitotic cells. At least 100 cells were analyzed for each condition. (B) HeLa cells were either mock treated or incubated for 1 hour with S23906 (5 μ M) or with SN-38 (200 nM). Cells were fixed with paraformaldehyde and processed for immunolabeling with an antibody directed against Thr68-phosphorylated Chk2. The fluorescence intensities were quantified by Metamorph analysis and are indicated in arbitrary units (a.u.). At least 200 cells were analyzed for each condition. The values represent the averages of three independent experiments. Standard deviations are indicated by error bars. (C) HeLa cells were either mock treated or incubated for 1 hour with S23906 (5 μ M) or with SN-38 (200 nM). Total protein extracts were prepared and analyzed by immunolabeling with antibodies directed against Ser1981-phosphorylated ATM, ATM, Thr68-phosphorylated Chk2 and Chk2. Total ATM and Chk2 were used as loading control. (D) HeLa cells were either mock treated or incubated for 1 hour with S23906 (5 μ M) or with SN-38 (200 nM). Soluble proteins were extracted with ice-cold CSK-lysis buffer for 5 minutes and the detergent-insoluble proteins were subjected to immunoblotting with antibodies directed against Rad50 or Mre11. Lamin B was used as a loading control. (E) HeLa cells were either mock treated or incubated for 1 hour with S23906 (5 μ M) or with SN-38 (200 nM). Soluble proteins were extracted with ice-cold CSK-lysis buffer for 5 minutes before fixation and immunolabeling with an Nbs1-directed antibody.

Figure 3: S23906 induces rapid activation of the ATR/Chk1 pathway in replicating cells

(A) HeLa cells were incubated with EdU for 30 minutes followed by 1 hour exposure to S23906 (5 μ M). Soluble proteins were extracted with ice-cold CSK-lysis buffer and the cells were labeled with a RPA70-directed antibody. (B) Same as above, except that the cells were immunolabeled with a RPA32-directed antibody. The merged image shows colocalization (in yellow) of RPA32 and EdU staining. (C) HeLa cells were either mock treated or incubated for 1 hour with S23906 (5 μ M) or SN-38 (200 nM). Total protein extracts were subjected to Western blot analysis using a RPA32-directed antibody. Lamin B was used as loading control. (D) HeLa cells were treated for 1 hour with S23906 (5 μ M). Soluble proteins were extracted with ice-cold CSK-lysis buffer for 5 minutes at 4°C before fixation and the cells were immunolabeled with antibodies directed against TopBP1 or RPA70. The merged image shows colocalization (indicated by arrows) of TopBP1 and RPA70. The dotted circles represent RPA70-negative cells. (E) HeLa cells were either mock treated or incubated for 1 hour with S23906 (5 μ M) or SN-38 (200 nM) followed by immunolabeling with an antibody directed against Ser317-phosphorylated Chk1. Fluorescence intensities were quantified by Metamorph analysis and are indicated in arbitrary units (a.u.). At least 200 cells were analyzed for each condition. Bars indicate the average intensity obtained for each condition. (F) HeLa cells were either mock treated or incubated for 1 hour with S23906 (5 μ M) or with SN-38 (200 nM). Total protein extracts were prepared followed by immunolabeling with antibodies directed against Ser317-phosphorylated Chk1 and Ser345-phosphorylated Chk1. Total Chk1 was used as loading control. (G) HeLa cells were incubated with S23906

(2 μM) for the indicated times followed by Western blot analysis with a Cdc25A-targeted antibody. β -actin was used as loading control.

Figure 4: The early induction of γ -H2AX following S23906 exposure is ATR-dependent

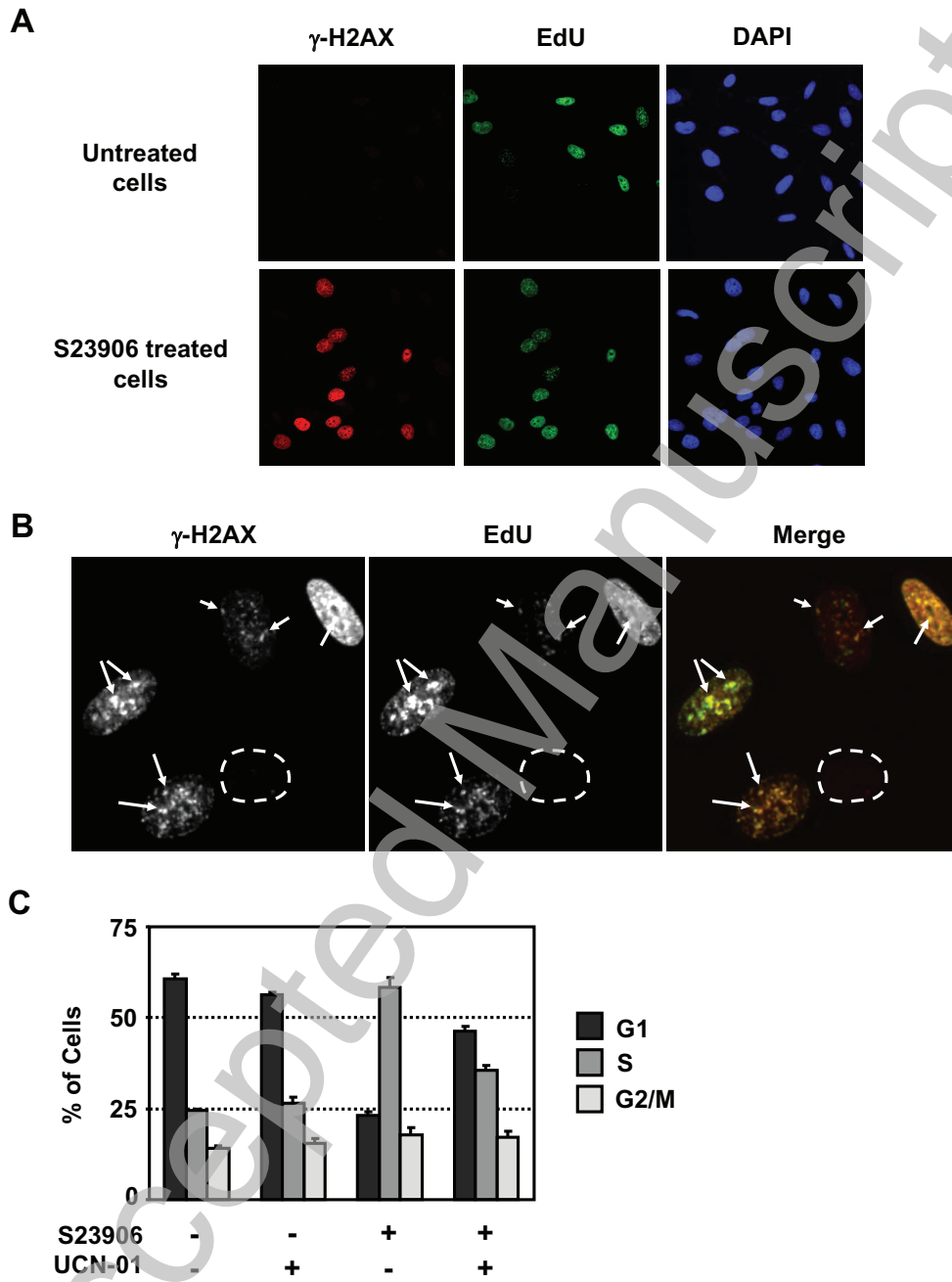
(A) Wild type donor (GM03657), ATR-Seckel donor (GM18367, ATR “low”) and ataxia telangiectasia (A-T) donor lymphoblastoid cells (GM03189, ATM deficient) were incubated for 1 hour in presence of S23906 (5 μM), followed by immunolabeling with a γ -H2AX-directed antibody. (B) Wild type donor (GM03657), ATR-Seckel donor (GM18367) and Ataxia telangiectasia (A-T) donor lymphoblastoid cells (GM03189) were incubated for 1 hour in presence of S23906 (5 μM). Cellular extracts were prepared and analyzed by immunoblotting with the indicated antibodies. (C) Wild type donor (GM03657), ATR-Seckel donor (GM18367) and Ataxia telangiectasia (A-T) donor lymphoblastoid cells (GM03189) were incubated for 1 hour in presence of the indicated concentrations of S23906 and subjected to single cell electrophoresis (the comet assay) under alkaline conditions. The circles indicate the levels of DNA damage in individual cells as determined by the Komet 5.5 software. At least 100 cells were analyzed for each condition. (D) Protein extracts were prepared from GM847 cells with inducible kinase-dead ATR incubated for 48 hours in absence (lane 1) or presence (lane 2) of doxycycline (5 $\mu\text{g}/\text{mL}$) followed by immunoblotting with an ATR-targeted antibody. Lamin B was used as loading control. (E) and (F) ATRkd-expressing cells were incubated in absence (light grey columns) or presence (dark grey columns) of doxycycline (5 $\mu\text{g}/\text{mL}$) for 48 hours followed by 1 hour exposure to the solvent control, to S23906 (5 μM) or to SN-38 (200 nM). The soluble proteins were extracted with ice-cold CSK-lysis buffer for 5 minutes at 4°C and the cells were labeled with γ -H2AX (panel E) or RPA70 (panel F)-directed antibodies. Fluorescence intensities were quantified by Metamorph analysis and are expressed in arbitrary units (a.u.). At least 200 cells were analyzed per condition. Values represent the averages of two independent experiments. Standard deviations are indicated by error bars. (G) ATR or ATM protein levels were knocked down by RNA interference in HeLa cells. 48 hours after transfection, cells were exposed for 1 hour to the solvent control or to S23906 (5 μM). Cellular extracts were analyzed by immunoblotting with the indicated antibodies. Scrambled siRNA (sc) was used as a negative control to measure the effect of our experimental siRNA duplexes versus background. (H) ATR or ATM levels were knocked down by RNA interference in HeLa cells. 48 hours after transfection, cells were exposed for 1 hour to the solvent control or to S23906 (5 μM). Cells were fixed and processed for γ -H2AX immunolabeling. Scrambled (sc) siRNA was used as a negative control. Fluorescence intensities were quantified by Metamorph analysis and are expressed in arbitrary units (a.u.). At least 200 cells were analyzed per condition. Values represent the averages of two independent experiments. Standard deviations are indicated by error bars.

Figure 5: ATR function influences the delayed response to S23906

(A) ATRkd-expressing GM847 cells were incubated in absence (- Dox) or presence (+ Dox) of doxycycline (5 $\mu\text{g}/\text{mL}$) for 48 hours followed by 1 hour exposure to S23906 (2 μM) and post-incubation in drug-free medium for the indicated times. Cells were processed for γ -H2AX immunolabeling and the fluorescence intensities were quantified by Metamorph analysis. The intensity of the γ -H2AX labeling is expressed in arbitrary units (a.u.). At least 200 cells were analyzed per condition.

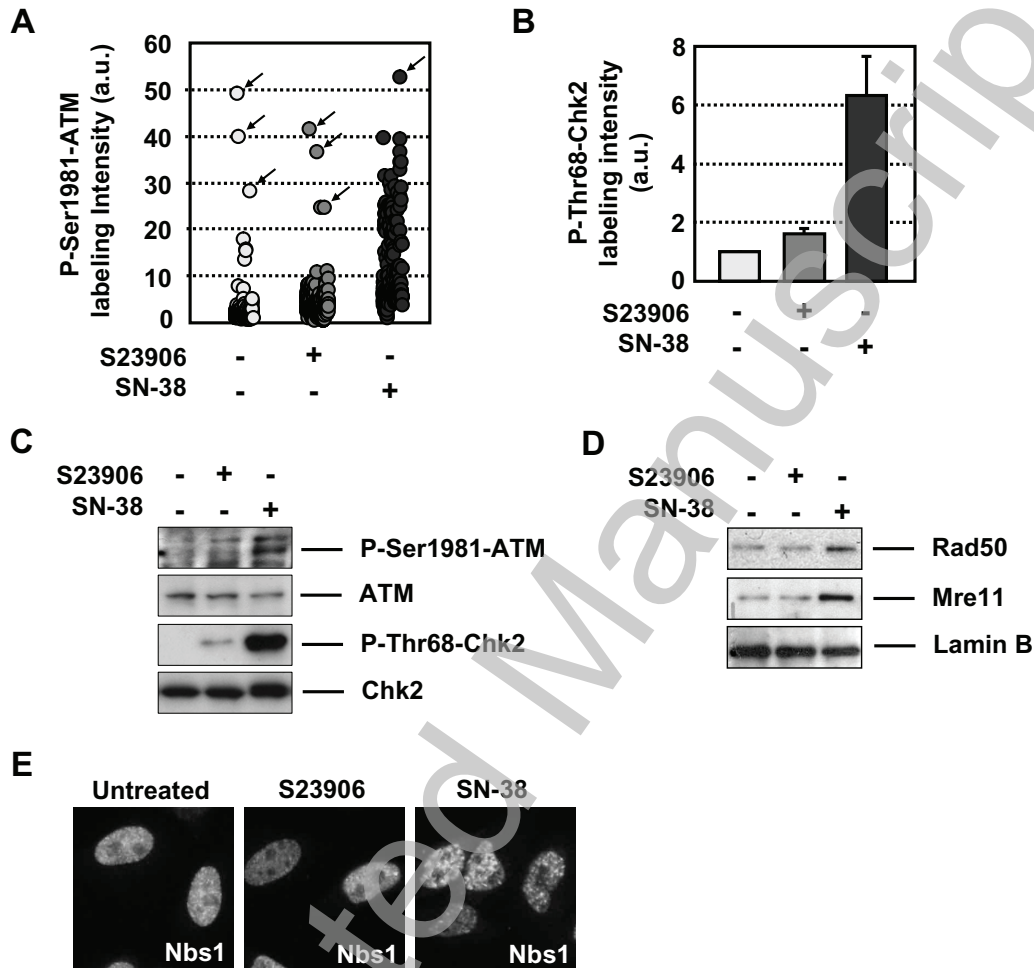
Values represent the averages of three independent experiments. Standard deviations are indicated by error bars when they exceed symbol size. **(B)** Cells were treated as outlined in A and prepared for immunoblot analysis with antibodies directed against Thr68-phosphorylated Chk2, total Chk2, Ser317-phosphorylated Chk1, total Chk1 and RPA32. **(C)** Cells were treated as outlined in A and processed for immunolabeling with an antibody directed against Thr68-phosphorylated Chk2. The fluorescence intensity of individual cells was quantified by Metamorph analysis and is indicated in arbitrary units (a.u.). At least 200 cells were analyzed per condition. Values represent the averages of two independent experiments. Standard deviations are indicated by error bars when they exceed symbol size. **(D)** Influence of ATR on the cell cycle distribution of S23906-treated cells. ATRkd-expressing cells were incubated in absence (1st and 2nd panels) or presence (3rd and 4th panels) of doxycycline (5 µg/mL) for 48 hours, incubated for 1 hour in the absence (1st and 3rd panels) or presence (2nd and 4th panels) of S23906 (1 µM), post-incubated for 12 hours in drug-free media and processed for cell cycle analysis. The values represent the averages of four independent experiments. Standard deviations are indicated by error bars. **(E)** GM847 cells with inducible ATRkd were incubated for 48 hours in the absence (- Dox) or presence (+ Dox) of doxycycline (5 µg/mL) and treated for 1 hour with the indicated concentrations of S23906. The cells were post-incubated for 5 generation doubling times in the absence (- Dox) or presence (+ Dox) of doxycycline and the fraction of viable cells was determined by the MTT assay. All values are average of three independent experiments, each done in duplicate. Standard deviations are indicated by error bars when they exceed symbol size. **(F)** GM847 cells were exposed to increasing concentrations of S23906 for 1 hour in the absence or presence of the Chk1/2 inhibitor AZD7762 (50 nM). The cells were post-incubated for 5 generation doubling times in drug-free media in the absence or presence of AZD7762 (50 nM) and the fraction of viable cells was determined by the MTT assay. All values are averages of two independent experiments each done in duplicate. Standard deviations are indicated by error bars when they exceed symbol size.

Figure 1



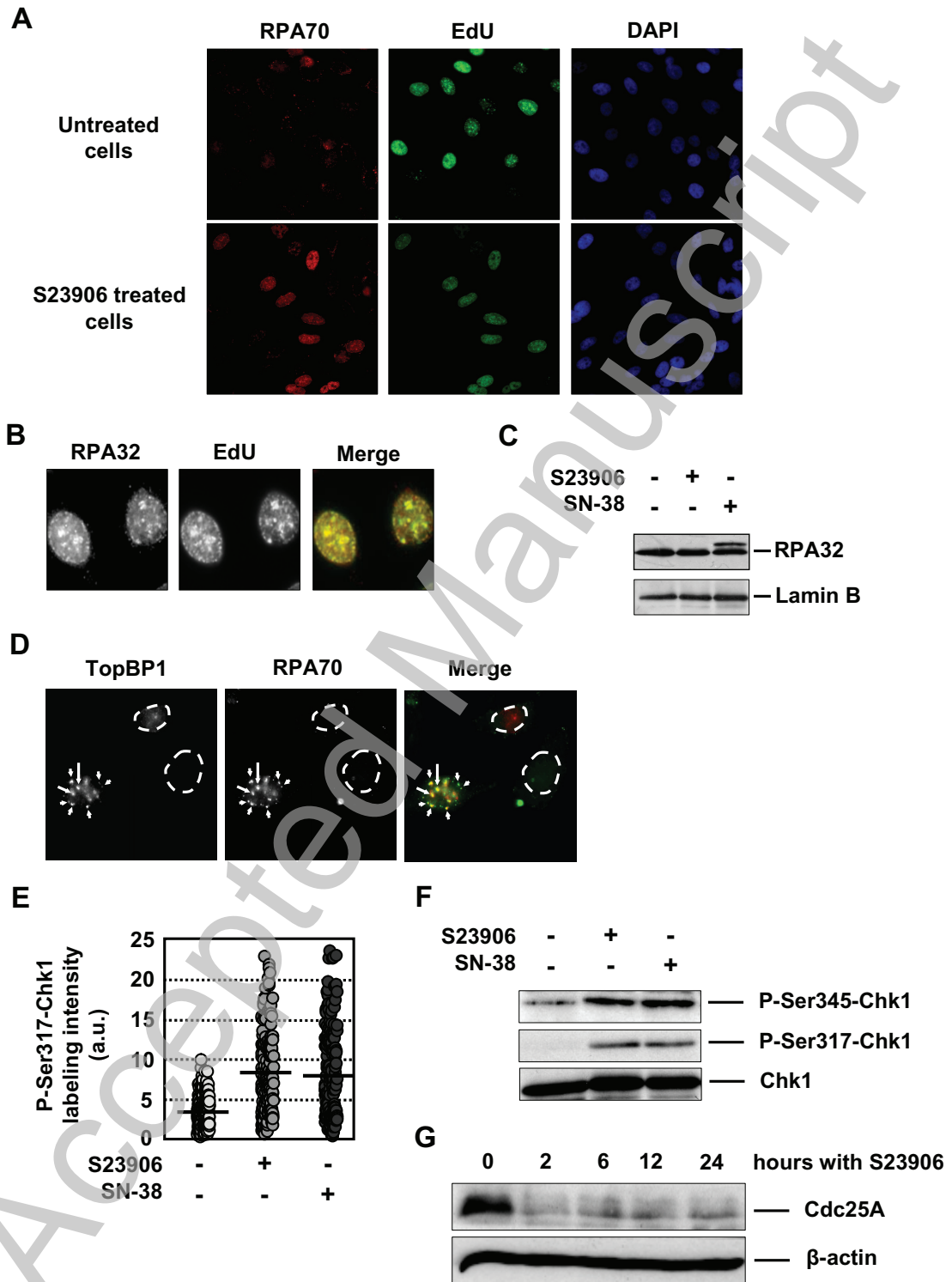
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Figure 2



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Figure 3



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Figure 4

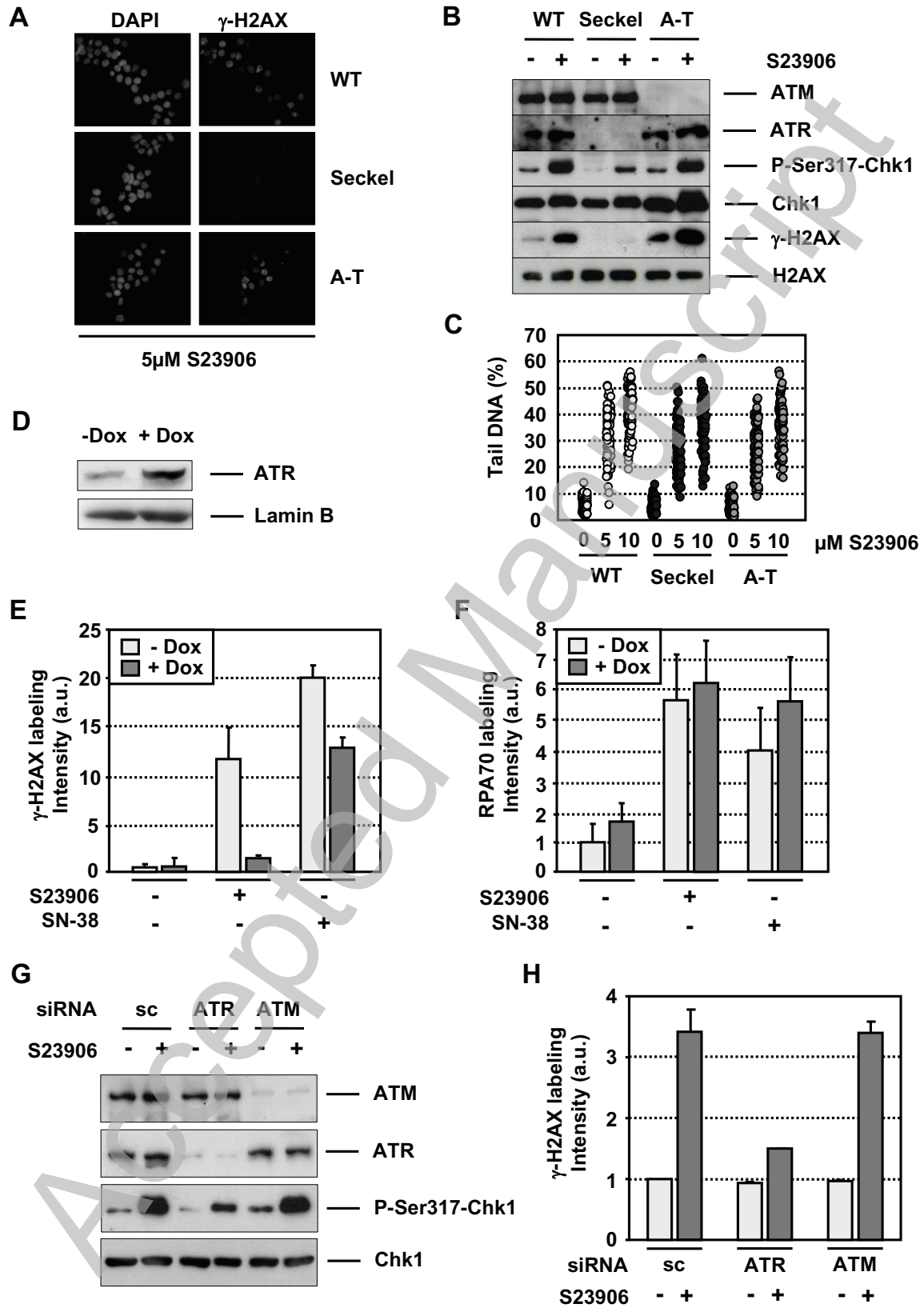
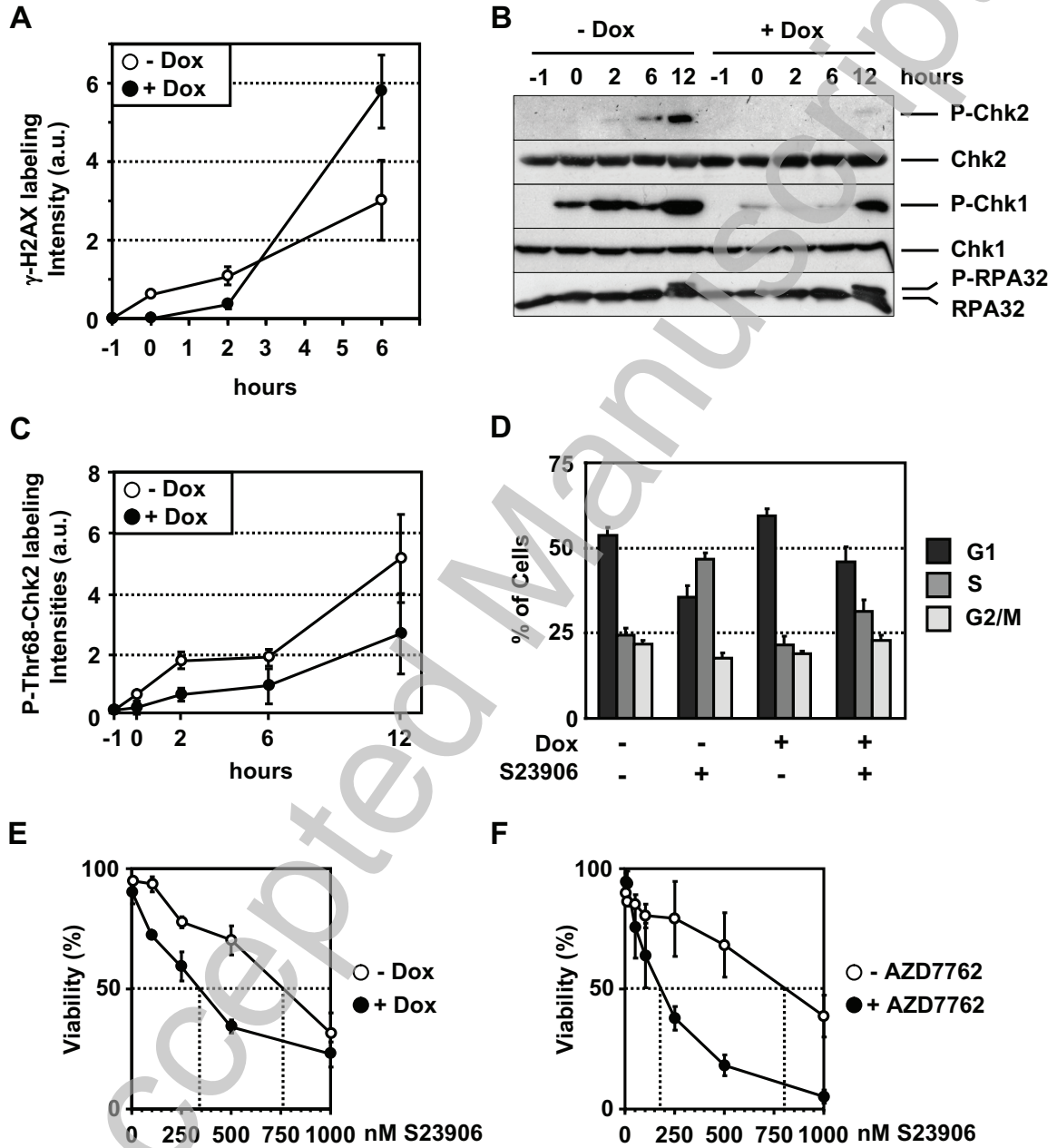


Figure 5



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