Ataxia telangiectasia and Rad3-related kinase drives both the early and the late DNA-damage response to the monofunctional antitumour alkylation S23906

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INTRODUCTION

Eukaryotic cells have developed numerous genome maintenance processes in order to co-ordinate DNA repair and cell-cycle progression. The major regulators of the DNA-damage response are the two PIKKs [PI3K (phosphoinositide 3-kinase)-related kinases], ATM [A-T (ataxia-telangiectasia) mutated] and ATR (ATM- and Rad3-related) [1,2]. ATM initiates the cellular response to DNA DSBs (double strand breaks) and is recruited to damaged sites by the MRN (Mre11-Rad50-Nbs1) complex via its interaction with the C-terminal domain of Nbs1 [3,4]. ATR, 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 ATRIP (ATR-interacting protein) to regions of RPA (replication protein A)-coated stretches of ssDNA (single-stranded DNA) 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 both have common phosphorylation substrates, such as γ-H2AX, and distinct substrates. For example, ATM phosphorylates and activates the distal transducer kinase Chk2, whereas 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 HU (hydroxyurea) 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 drug exposure for 1 h [22]. Conversely, ATR activation following IR (ionizing radiation) 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 (DNA-activated protein kinase) [25,26]. Similarly, RPA32 phosphorylation following UV or HU exposure, which is a slower process that probably occurs after replication fork collapse, requires ATR, ATM and MRN [27–30].

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. In the present study, we characterize the DNA-damage response to the antitumour acrycine derivative S23906, which forms monofunctional adducts with guanine residues in the minor groove of DNA. S23906 exposure is accompanied by specific recruitment of RPA (replication protein A) 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 Ras3-related) 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 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 co-ordinator of the DNA-damage response to S23906, and provide a mechanistic rationale for combinations of S23906 and similar agents with checkpoint abrogators.

Key words: checkpoint control, DNA alkylator, DNA-damage response, DNA replication.
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, such as BRCA2 (breast cancer early-onset 2) 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 interfere 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 antitumour 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, although 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 ATR, ATM or DNA-PK [44]. In contrast, adozelesin activates the ATR/Chk1 pathway, whereas irofulven specifically induces the ATM/Chk2 signalling 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 the present 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 adducts by the nucleotide-excision repair of the primary adducts by the nucleotide-excision repair pathway leads to formation of secondary DNA lesions in the 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]. The results of the present study 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 co-ordinator 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.

**Chemicals**

The acronycine derivative S23906 [cis-1,2-diaceetoxy-3,14-dihydro-3,3,14-trimethyl-6-methoxy-7H-benz(b)pyrazone(3,2-d)]acrigin-7-one] was obtained from Institut de Recherches Servier (Croissy sur Seine, France), whereas SN-38 (7-ethyl-10-hydroxy camptothecin), the active metabolite of camptothecin, was purchased from Abatra Technology. Doxycycline and UCN-01 were purchased from Sigma–Aldrich. AZD7762 was purchased from Axon Medchem. Protease inhibitors were obtained from Roche Diagnostics.

**Antibodies**

The phosho-Thr^68-Chk2 (#2661), Chk2 (clone 1C12, #3440), phospho-Ser^17-Chk1 (#2344), phospho-Ser^345-Chk1 (#2341), Chk1 (clone 2G1D5, #2360), phospho-Ser^981-ATM (clone 10H11.E12, #4526), ATM (clone D2E2, #2873), RPA32 (clone 4E4, #2208) and RPA70 (#2267) -directed antibodies were purchased from Cell Signaling Technology. The TopBP1 (#611874) and the β-actin-directed antibodies (clone AC-15, #A-54421) were purchased from BD Biosciences and Sigma–Aldrich respectively. The H2AX (#07-627) and γ-H2AX (#05-636) -directed antibodies were purchased from Millipore. The Mre11 (clone 12D7), Rad50 (clone 13B3) and Nbs1-directed antibodies were purchased from Novus Biologicals. Finally, the lamin B (clone M-20, #sc-6217), ATM (clone E-14, #sc-23736) and Cdc25A (clone F6, #sc-7389) -directed antibodies were purchased from Santa Cruz Biotechnology. HRP (horseradish peroxidase) and fluorescent dye-conjugated antibodies were purchased from Jackson ImmunoResearch.

**Cell-cycle analysis**

For cell-cycle analysis, experiments were carried out as described previously [55]. Briefly, HeLa cells were exposed to 1 μM S23906 for 1 h followed by 6 h in drug-free medium. When...
indicated, cells were co-incubated with 100 nM UCN-01 and 1 μM S23906 for 1 h and post-incubated for 6 h in the presence of 100 nM UCN-01. ATRkd-expressing cells were incubated in the absence or presence of 5 μg/ml doxycycline for 48 h prior to cell-cycle analysis. ATRkd cells were then exposed to 1 μM S23906 for 1 h followed by 12 h 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 [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Cells were washed and fixed with 70 % ethanol. Cells were resuspended in PBS and incubated for 30 min at room temperature (20°C) in a solution containing 5 μg/ml DAPI (4',6-diamidino-2-phenylindole; Sigma–Aldrich) and 100 μg/ml RNase (Sigma–Aldrich). Samples were analysed with a LSR II flow cytometer and DIVA software (BD Biosciences).

**Immunofluorescence and microscopy**

Cells were incubated with different concentrations of S23906 at 37°C for the indicated times. For the short exposure time (1 h), 5 μM S23906 was used because it gave a strong and very reproducible DNA-damage signal by 1 h in HeLa, lymphoblastoid or ATRkd cells. When cells were post-incubated for up to 12 h (Figures 5A and 5C), the concentration used was decreased to 2 μM S23906 to avoid unspecific labelling 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 X-100, 50 mM Hepes (pH 7.4), 30 mM sucrose and protease inhibitors] and kept at 4°C for 5 min before fixation in 4% paraformaldehyde (Electron Microscopy Sciences). The antigens were revealed using the indicated primary antibodies. DNA was counterstained with DAPI. Replicating cells were labelled by incorporation of EdU (5-ethyl-2'-deoxyuridine) (Click-iTTM EdU Alexa Fluor® 488 Imaging Kit, #C10337, Invitrogen) for 30 min prior to drug addition. EdU labelling 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 using MetaMorph software (Universal Imaging). The background over non-cellular regions was subtracted. At least 100 cells were analysed per sample. Values represent the mean 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 min at 4°C in SDS/PAGE loading buffer. Alternatively, cells were washed twice in PBS and resuspended in ice-cold CSK-lysis buffer and kept at 4°C for 5 min. Soluble proteins were separated from detergent-resistant proteins by centrifugation at 1500 g for 5 min. For the short exposure time (1 h), 5 μM S23906 was used because it gave a strong and very reproducible DNA-damage signal by 1 h for all cell lines tested. When cells were post-incubated for up to 24 h (Figures 3G and 5B), the concentration used for S23906 was decreased to 2 μM to avoid unspecific labelling due to the induction of cell death [52]. Proteins were resolved on linear-gradient SDS/PAGE (5–15 %) and blotted on to nitrocellulose membranes (Bio-Rad). Membranes were saturated by TBST-milk [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.5 % Tween 20 and 5 % dehydrated skimmed milk] and the antigens were revealed by immunolabelling. Antigens were detected using an enhanced chemiluminescence kit (Amersham Biosciences).

**Depletion of ATR or ATM by siRNA (small interfering RNA)**

siRNA transfections were performed accordingly to the manufacturer’s instructions with HiPerfect transfection reagent (Qiagen) and 10 nmol/l final concentration of oligonucleotides. siRNA duplexes 25 nucleotides in length were purchased from Invitrogen. The siRNA target sequence of ATR is 5'-CAUUCAGG-AGUUGCUUUCUAUUU-3'. The siRNA target sequence of ATM is 5'-UAAGCAGAUCUUAGACUAGCU-3'. Scrambled siRNA (Stealth RNAi™ siRNA Negative Control LO GC) was purchased from Invitrogen. Transfection mixtures were removed after 6 h and cells were incubated with complete medium for 48 h 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 using the MTT viability assay, as described previously [57]. All values are means of at least three independent experiments, each performed in duplicate. ATRkd-expressing cells were incubated in the absence or presence of 5 μg/ml doxycycline for 48 h prior to the start of the viability assays. ATRkd-expressing cells were then exposed to S23906 for 1 h followed by five 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 h in the absence or presence of 50 nM AZD7762. Then the cells were washed and incubated for five generation doubling times in the absence or presence of 50 nM AZD7762.

**Single-cell gel electrophoresis (comet assay)**

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

**RESULTS**

**S23906 specifically induces an early DNA-damage response in replicating cells**

To establish the relationship between early γ-H2AX induction and the replication status, HeLa cells were labelled with EdU, a thymidine analogue, prior to treatment with S23906. Although no γ-H2AX signal could be observed in the absence of drug, S23906 exposure was accompanied by an important increase in γ-H2AX labelling. 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 a drug concentration as high as 5 μM (Figure 1A and Supplementary Figure S1 at http://www.BiochemJ.org/bj/437/bj4370063add.htm). 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.
experiments. In medium with 100 nM UCN-01 (fourth column). Values are means ± S.D. for four independent experiments.

**Figure 1** S23906 selectively targets replicating cells

Proliferating HeLa cells were incubated with EdU for 30 min followed by 1 h exposure to S23906 (5 μM). Cells were fixed and processed for EdU and γ-H2AX staining, and the DNA was counterstained with DAPI. (A) Increased magnification of the cells shown in (A). The merged image illustrates the co-localization of EdU and γ-H2AX (indicated with arrows). The dotted circle represents a non-replicating cell. (B) 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 h (first column), or exposed to 100 nM UCN-01 for 7 h (second column), or S23906 (1 μM) for 1 h followed by 6 h post-incubation in drug-free medium (third column) or co-incubated with 100 nM UCN-01 and 1 μM S23906 for 1 h followed by 6 h post-incubation in medium with 100 nM UCN-01 (fourth column). Values are means ± S.D. for four independent experiments.

S23906 exposure was accompanied by rapid modification of the cell-cycle distribution. Indeed, 1 h exposure to S23906 (1 μM) followed by 6 h post-incubation in drug-free medium was accompanied by a marked enrichment of the S-phase fraction (Figure 1C, compare the first and third columns), 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 third and the fourth columns). 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]. Taken 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 Ser681 after 1 h exposure to 5 μM S23906 or to 200 nM SN-38 (Figure 2A). The results show that S23906 does not modify the phosphorylation status of ATM compared with 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 Ser681 phosphorylation of mitotic cells was not influenced by S23906 exposure (Figure 2A, indicated by arrows, and Supplementary Figure S2 at http://www.BiochemJ.org/bj/437/bj4370063add.htm). In agreement with the results for ATM, S23906 had only marginal influence on the phosphorylation of Chk2 on Thr68 (Figure 2B, compare the first and the second columns) in comparison with SN-38-exposed cells (Figure 2B, third column). Thus, even at high drug concentrations, no activation of the ATM pathway can be detected after 1 h exposure to S23906.

Because phospho-specific 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 with 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). Taken 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 ssDNA that are generated by decoupling of helicase and polymerase activities at stalled replication forks. Short (1 h) S23906 exposure was accompanied by strong chromatin recruitment of RPA70/RPA1, one of the three RPA subunits (Figure 3A), which was selective for replicating cells (Figure 3A and Supplementary Figure S3 at http://www.BiochemJ.org/bj/437/bj4370063add.htm). 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 with 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 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 exposure to S23906 was accompanied by recruitment of TopBP1 to the RPA-coated regions, as shown by immunocytochemistry (Figure 3D, indicated with arrows). This was accompanied by rapid activation of ATR,
S23906 is a potent inducer of replication stress

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

(A) HeLa cells were mock-treated or exposed for 1 h to S23906 (5 μM) or SN-38 (200 nM). Cells were fixed and processed for immunolabelling 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 analysed for each condition. (B) HeLa cells were either mock-treated or incubated for 1 h with S23906 (5 μM) or with SN-38 (200 nM). Cells were fixed with paraformaldehyde and processed for immunolabelling 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 analysed for each condition. Values are means ± S.D. for three independent experiments. (C-D) HeLa cells were either mock-treated or incubated for 1 h with S23906 (5 μM) or with SN-38 (200 nM). Total protein extracts were prepared and analysed by immunoblotting with antibodies directed against Ser1981-phosphorylated ATM, ATM, Thr68-phosphorylated Chk2 and Chk2. Lamin B was used as a loading control. (E) HeLa cells were either mock-treated or incubated for 1 h with S23906 (5 μM) or with SN-38 (200 nM). Soluble proteins were extracted with ice-cold CSK-lysis buffer for 5 min before fixation and immunolabelling with a Nbs1-directed antibody.

as indicated by the formation of phosphorylated Chk1 on both Ser317 and Ser345 (Figures 3E and 3F) which was selective for actively replicating cells (Supplementary Figures S4 and S5 at http://www.BiochemJ.org/bj/437/bj4370063add.htm). 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-Seqel donor, ATR ‘low’) and GM03189 (ATM-deficient). Exposure of normal and ATM-deficient cells to S23906 was accompanied by formation of comparable levels of γ-H2AX, whereas no γ-H2AX staining was observed for the ATR-Seqel cells (Figures 4A and 4B). Accordingly, Chk1 phosphorylation induced in response to S23906 was strongly diminished in ATR-Seqel cells compared with normal and ATM-deficient cells (Figure 4B). Importantly, the lack of γ-H2AX staining and Chk1 phosphorylation in the ATR-Seqel cells was not due to differences in the induction of DNA damage, since exposure to S23906 for 1 h resulted in comparable levels of DNA damage for the three cell lines, as determined using the alkaline comet assay (Figure 4C).

In agreement, overexpression of a kinase-dead form of ATR (ATRkd) in GM847 human fibroblasts (Figure 4D, right-hand lane) 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, although 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, RNAi (RNA interference) experiments were carried out in HeLa cells. By 48 h after siRNA transfection, the 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 Ser317 was markedly reduced following S23906 treatment (Figure 4G). In clear contrast, ATM knockdown did not influence the ability of the cells to induce Chk1 phosphorylation (Figure 4G). Moreover, ATR knockdown, but not ATM knockdown, 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 h, this was only a transient effect (Figure 5A). Indeed, when these cells were post-incubated in drug-free medium, detectable γ-H2AX appeared after 2 h, and by 6 h 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 also interfered with the late phosphorylation of Thr68 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 cell-cycle progression, GM847 cells overexpressing or not ATRkd were exposed to S23906 for 1 h followed by 12 h post-incubation in drug-free medium and processing for flow cytometry analysis. The results show that attenuation of ATR activity interferes with the S23906-induced...
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 A-T donor lymphoblastoid cells (GM03189, ATM deficient) were incubated for 1 h in the presence of S23906 (5 μM), followed by immunolabelling with a γ-H2AX-directed antibody. (B) Wild-type donor (GM03657), ATR-Seckel donor (GM18367) and A-T donor lymphoblastoid cells (GM03189) were incubated for 1 h in the presence of S23906 (5 μM). Cellular extracts were prepared and analysed by immunoblotting with the antibodies indicated. (C) Wild-type donor (GM03657), ATR-Seckel donor (GM18367) and A-T donor lymphoblastoid cells (GM03189) were incubated for 1 h in the 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 analysed for each condition. (D) Protein extracts were prepared from GM047 cells with inducible ATRkd incubated for 48 h in the absence (lane 1) or presence (lane 2) of doxycycline (5 μg/ml) followed by immunoblotting with an ATR-targeted antibody. Lamin B was used as a loading control. (E) and (F) ATRkd-expressing cells were incubated in the absence (light grey columns) or presence (dark grey columns) of doxycycline (5 μg/ml) for 48 h followed by 1 h 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 min at 4°C and the cells were labelled with γ-H2AX (E) or RPA70 (F) -directed antibodies. Fluorescence intensities were quantified by Metamorph analysis and are expressed in arbitrary units (a.u.). At least 200 cells were analysed per condition. Values are means ± S.D. for two independent experiments. (G) ATR or ATM protein levels were knocked down by RNAi in HeLa cells. At 48 h after transfection, cells were exposed for 1 h to the solvent control or to S23906 (5 μM). Cellular extracts were analysed by immunoblotting with the antibodies indicated. Scrambled siRNA (sc) was used as a negative control to measure the effect of our experimental siRNA duplexes compared with background. (H) ATR or ATM levels were knocked down by RNAi in HeLa cells. At 48 h after transfection, cells were fixed and analysed for γ-H2AX immunolabelling. 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 analysed per condition. Values are means ± S.D. for two independent experiments.
S-phase arrest (Figure 5D, compare the second and fourth columns). Interestingly, attenuation of functional ATR was also accompanied by a 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 such as Chk1 and Chk2 (Figures 5B and 5C). Therefore inhibition of the distal checkpoint kinases rather than the proximal kinases (e.g. ATR and ATM) might be an alternative strategy to improve the cytotoxic activity of S23906. 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 5(F), AZD7762 potentiated the cytotoxicity of S23906 approximately 5-fold. A similar effect of AZD7762 was observed for HeLa cells (Supplementary Figure S6 at http://www.BiochemJ.org/bj/437/bj4370063add.htm). 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 at http://www.BiochemJ.org/bj/437/bj4370063add.htm).
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 h, 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.

In the present report, 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 ssDNA regions are formed at the stalled replication forks, probably due to uncoupling of helicase and polymerase activities. Consistent with the formation of ssDNA, the initial DNA-damage response is strongly, if not exclusively, ATR-dependent. In contrast, MRN and ATM have no detectable influence at early time points, even at high drug concentrations (e.g. 5 μM).

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-exclusive 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 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 such as 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 though γ-H2AX and co-operate 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 with what has been reported for camptothecin, UV adducts and irofulven [21,22,46], the ATM/Chk2 pathway is activated relatively late following S23906 exposure. RPA32 hyperphosphorylation is also a late phenomenon compared with camptothecin and adozelen [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]. Taken 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 ATRkd) 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 tumour 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 results suggest that combining S23906 with checkpoint abrogators might increase its cytotoxic activity. In agreement, we show in the present study 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 and 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.

Taken together, the results of the present study 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 Soares carried out the immunofluorescence studies, the image analysis and the viability assays. She also 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. Célina Rocca participated in the cell-cycle analysis and the comet assay. Renata Matuo participated in the immunoblotting assays and the viability assays. João Henriques participated in the design of the study. Annette Larsen conceived the study, and participated in its design and co-ordination. Annette Larsen also helped to draft the manuscript. Alexandre Escargueil conceived, designed and co-ordinated the study, and drafted the manuscript.

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S23906 is a potent inducer of replication stress


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SUPPLEMENTARY ONLINE DATA

Ataxia telangiectasia mutated- and Rad3-related kinase drives both the early and the late DNA-damage response to the monofunctional antitumour alkylator S23906

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Figure S1 Phosphorylation of histone H2AX after S23906 exposure is selective for replicating cells

Proliferating HeLa cells were incubated with EdU for 30 min followed by 1 h exposure to S23906 (5 μM). Cells were fixed and processed for staining of EdU and γ-H2AX. The fluorescence intensities were quantified by Metamorph analysis and are indicated in arbitrary units (a.u.). ρ denotes the correlation coefficient between the intensities of EdU and γ-H2AX.

Figure S2 ATM is not activated in response to S23906

HeLa cells were either mock-treated (top panels), or incubated for 1 h with 5 μM S23906 (middle panels) or with 200 nM SN-38 (bottom panels). Cells were fixed and processed for immunolabelling with an antibody directed against Ser1981-phosphorylated ATM to determine the levels of ATM autophosphorylation. The merged images correspond to the framed regions represented in the panels for DAPI and phospho-Ser1981-ATM. Mitotic ATM phosphorylation was observed under all conditions, whereas only SN-38 treatment induced the formation of Ser1981-phosphorylated ATM in interphase cells.

Figure S3 Chromatin recruitment of RPA70 after S23906 exposure is selective for replicating cells

HeLa cells were incubated with EdU for 30 min followed by 1 h exposure to S23906 (5 μM). Soluble proteins were extracted with ice-cold CSK-lysis buffer and the cells were labelled with an RPA70-directed antibody. The fluorescence intensities were quantified by Metamorph analysis and are indicated in arbitrary units (a.u.). ρ denotes the correlation coefficient between the intensities of EdU and RPA70.

Figure S4 Chk1 phosphorylation on Ser317 is selective for replicating cells

Proliferating HeLa cells were incubated with EdU for 30 min followed by 1 h exposure to S23906 (5 μM). Cells were fixed and processed for labelling of EdU and Ser317-phosphorylated Chk1.

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**Figure S5**  Chk1 phosphorylation on Ser<sup>317</sup> after exposure to S23906 is selective for replicating cells

Proliferating HeLa cells were incubated with EdU for 30 min followed by 1 h exposure to S23906 (5 μM). Cells were fixed and processed for staining of EdU and Ser<sup>317</sup>-phosphorylated Chk1. The fluorescence intensities were quantified by Metamorph analysis and are indicated in arbitrary units (a.u.). ρ denotes the correlation coefficient between the intensities of EdU and Ser<sup>317</sup>-phosphorylated Chk1.

**Figure S6**  AZD7762 increases the cytotoxicity of S23906

Proliferating HeLa cells were exposed to different concentrations of S23906 for 1 h in the absence (○) or presence (●) of AZD7762 (50 nM) followed by 120 h post-incubation in the absence or presence of AZD7762. The cellular viability was determined using the MTT assay. Values are means ± S.D. for two independent experiments, each performed in duplicate.

**Figure S7**  AZD7762 increases the cytotoxicity of SN-38

Proliferating HeLa cells were exposed to different concentrations of SN-38 in the absence (○) or presence (●) of AZD7762 (50 nM). The cellular viability was determined using the MTT assay. Values are means ± S.D. for two independent experiments, each performed in duplicate.