The DNA damage checkpoint is activated during residual tumour cell survival to methotrexate treatment as an initial step of acquired drug resistance

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In the process of acquired drug resistance, the absence of tumour cell subpopulations already resistant before treatment implies an initial adaptive stage of cell growth following drug exposure that, under the selective pressure of the drug, allows the emergence of stably resistant cell variants. Here, we show that p53-defective HT-29 colon cancer cells overcome methotrexate-induced cell death owing to DNA damage checkpoint-mediated cell survival at the adaptive stage that precedes stable resistance acquisition. HT-29 cell cycle progression was dramatically delayed in the presence of a lethal dose of methotrexate, leading to DNA damage during S-phase transition and to cell death as treated cells progressed to G_2 and M phases. As a result, the DNA damage checkpoint was induced as indicated by the presence of activated phosphorylated forms of checkpoint proteins Chk1 and Rad9. As we recently described, in-vitro resistance to methotrexate occurs without cell subpopulations already resistant before treatment, hence resistance is acquired through a multistep process that includes an early stage of transient cell survival. Our present results showed that this acute cell survival stage was due to a minor percentage of cells that could complete the first division cycle after drug exposure. Cell survival was enhanced by drug withdrawal during S-phase transition and suppressed if drug withdrawal was followed by treatment with the checkpoint-inhibitor drug caffeine. These results thus point to checkpoint-mediated transient adaptation as a target to prevent the emergence of acquired resistance to methotrexate. ***Anti-Cancer Drugs*** **17:1171–1177 © 2006 Lippincott Williams & Wilkins.**

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Introduction

The process of tumour drug resistance is responsible for many therapeutic failures in the treatment of cancer and may be considered one of the main consequences of tumour progression to increased malignancy. Two types of drug resistance exist, depending on the mechanism used by tumours to undergo drug insensitization [1]. First, inherent resistance occurs in tumour cell populations already resistant before treatment; i.e. cells less prone to drug-induced apoptosis as a result of tumour progression to apoptosis-defective phenotypes [2]. Similarly, certain extracellular conditions may favour drug insensitization as inherent resistance, e.g. the presence of extracellular nucleosides that salvage nucleotide synthesis in anti-metabolite-treated cells [1]. Second, acquired resistance occurs when tumour cell populations lacking resistant cells before treatment give rise to resistant genetic variants as a consequence of the treatment; such acquired resistance is thus selected by the pressure of treatment amid the sensitive population and is permanently expressed in the tumour because of its genetic nature.

Methotrexate (MTX) is an anticancer drug that shows therapeutic efficacy in the treatment of osteosarcomas and lymphomas, but some of these tumours undergo resistance and others, such as colon cancer, appear refractory to MTX antitumoral effects [3]. While several genetic mechanisms of MTX resistance have been identified both *in vivo* and *in vitro* [4], it is still obscure to what extent inherent versus acquired resistance is responsible for the failure of some MTX-based therapies. On the other hand, colon cancer cell lines display high

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sensitivity *in vitro* to MTX and provide experimental models suitable to investigate the mechanisms underlying the expression of drug resistance phenotypes. In particular, the HT-29 colon cancer in vitro model of resistance to MTX is a paradigm of the complex mechanisms of colon cancer cell resistance to antimetabolites [5]. We have recently characterized the early stages of the process of resistance acquisition to MTX using HT-29 colon cancer cells. We found that resistance acquisition is preceded by an adaptive cell survival stage, in a minor fraction of cells, early during treatment and coincides with the peak of MTX-induced cell death [6]. No resistant subpopulations are present in the HT-29 cell line before exposure to the drug and the mechanism of the adaptive cell survival phenomenon is yet poorly characterized. Of note, the minor HT-29 cell population that overcomes MTX treatment does not exert permanent resistant features but, instead, needs to undergo through a process of high cell renewal in the continuous presence of MTX before stably resistant cell clones appear [7]. Therefore, the HT-29 model of resistance to MTX provides a process of complex cell population dynamics, including adaptive and selection responses to treatment, that models tumour cell progression to acquired drug resistance, such as resistant tumours arising from minimal residual disease.

MTX inhibits reversibly the dihydrofolate reductase gene (*DHFR*), thus blocking the folate cycle required for the synthesis of thymidilate and purines [1]. Secondly to the nucleotide starvation, MTX treatment results in apoptosis induction and/or clastogenicity, depending on the cancer cell genotype [1]. Many drugs used to treat cancer are intended to interfere with the tumour cell division process and cell cycle checkpoints exert a role in the tumour cell response to therapy [8–12]. These checkpoints ensure that cell cycle progression takes place orderly and without errors; in the presence of problems, checkpoint activation results in cell cycle block to allow repair or to get rid of damaged cells by apoptosis. Moreover, checkpoints are frequently altered in tumour cells, so that these alterations may be determinant in their cell cycle response to drug treatment [13]. In this context, MTX and other antimetabolite drugs induce intracellular nucleotide starvation. While some inhibitors of ribonucleotide synthesis elicit *TP53*-dependent cell cycle arrest, e.g. PALA, inhibitors of dNTP synthesis, such as MTX, result in the arrest at the G_1/S boundary in a manner independent of the *TP53* status [1,14,15]. *TP53* deficiency is associated with inappropriate transit through S phase and DNA breakage, which, in MTX-treated cells, is a requirement for gene amplification and acquisition of resistance [1]. Moreover, the mechanisms of colon cancer cell resistance to MTX are largely dependent on the nature of their genetic instability, which is known to arise on particular altered checkpoints [16]. As MTX targets a key metabolic pathway of the cell cycle, i.e. *de novo* nucleotide synthesis, we aimed at understanding whether cell cycle checkpoints impinge on the acute cell survival response to MTX in the HT-29 cell line. Although MTX employs genotoxicity as part of its antitumoral activity [1], the cell cycle response of tumour cells in terms of cell sensitivity and resistance to the drug had never been addressed before. Within the context of HT-29 cell population dynamics of adaptation and resistance to MTX treatment, we obtained evidence indicating that acute cell survival to MTX, i.e. the initial step of the drug resistance acquisition process [6], is mediated by a checkpoint response that involves the DNA damage checkpoint.

Materials and methods

Cell culture and treatments

HT-29 cells were routinely seeded at 2×10^4 cells/cm^2 and cultured using Dulbecco's modified Eagle's medium medium, supplemented with 10% fetal bovine serum, at 37°C with 7.5% CO_2. Where indicated, 0.1 µmol/l MTX (Almirall, Barcelona, Spain), 10 mmol/l caffeine (Sigma, St Louis, Missouri, USA), or both, were added to the cells. Cells were first synchronized by maintaining cultures at confluence for 6 days, so G_0 arrest was induced, and then they were released by reseeding at 2×10^4 cells/cm^2. Clonogenic assays were performed by seeding cell suspensions at a density of 10 cells/cm^2 in standard culture medium; these cultures were maintained for 10 days, then washed with phosphate-buffered saline (PBS) and fixed in 2% formaldehyde, stained with Coomassie blue, and the colonies formed were counted with the naked eye.

Antibodies and Western blot analysis

Cells were harvested, washed with PBS and then lysed in denaturing buffer (6 mol/l urea, 50 mmol/l Tris–HCl, pH 6.8, 1% sodium dodecyl sulphate) by mild sonication on ice for 5 s using a UP100H ultrasonic processor adjusted at 70% of amplitude (Hielscher Ultrasound Technology, Teltow, Germany). Whole-cell extracts were subjected to electrophoresis in 12% sodium dodecyl sulphate–polyacrylamide gels and transferred to nitrocellulose membranes. Mouse monoclonal anti-phospho-Histone H2AX (S139) antibody clone JBW301 (Upstate, Charlottesville, Virginia, USA), anti-β-actin clone AC-15 (Sigma) and anti-phospho-Chk1 (Ser345) (Cell Signaling, Danvers, Massachusetts, USA) were used following the manufacturer's instructions. Rabbit anti-human Rad9 antibody was used at 1:2000 dilution [17]. Reactions were revealed by chemiluminescence, using the SuperSignal West Pico detection kit (Pierce, Rockford, Illinois, USA).

Immunofluorescence assay

Cells growing on coverslips were fixed by immersion in 50% methanol–50% acetone for 2 min. Then, they were permeabilized using 0.5% NP-40 in PBS for 10 min and blocked using 0.5% bovine serum albumin in PBS for

20 min. Mouse monoclonal anti-phospho-Histone H2AX (S139) antibody was diluted 1:500 in 0.5% bovine serum albumin and incubated for 1 h at room temperature. After a brief wash with PBS, samples were incubated with secondary rhodamine-conjugated goat anti-mouse antibodies (Jackson Immunoresearch, West Grove, Pennsylvania, USA) for 1 h at room temperature. Before visualization under the microscope, Vectashield mounting medium for fluorescence with DAPI stain was used (Vector Laboratories, Burlingame, California, USA).

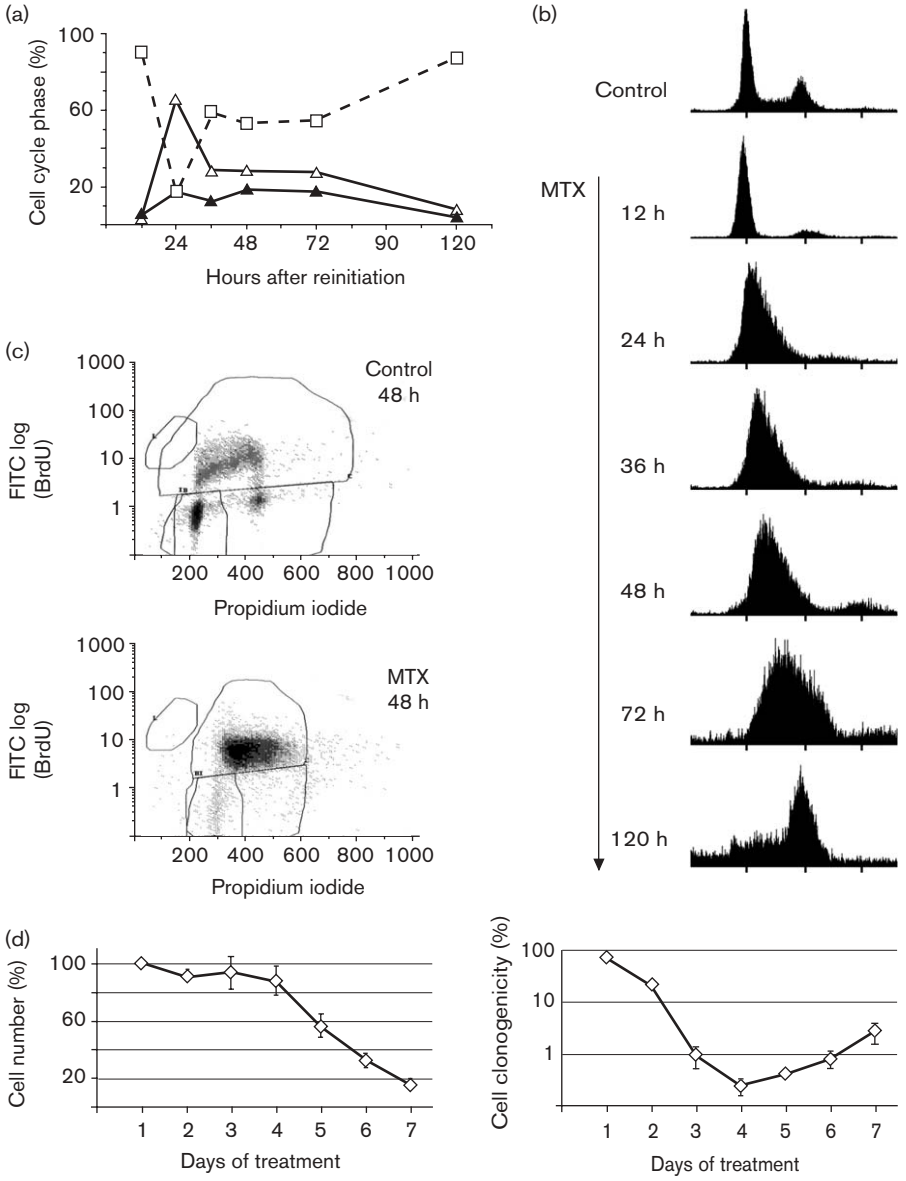
Results and discussion

HT-29 tumour cells contain a *TP53* arginine-273 to histidine mutation that inactivates its function; similarly, *TP53* loss of function has also been lost in about half of colorectal cancer [18]. As the kinetics of MTX-induced genotoxicity during the cell cycle under defined tumour genetic backgrounds (such as *TP53*-mutated HT-29 tumour cells) are largely unknown, we first characterized the HT-29 cell cycle under MTX treatment. We synchronized HT-29 cells in G_0 by maintaining cultures in a confluent state for 1 week, approximately, and released them from the arrest by seeding at subconfluent density. Using this protocol, HT-29 cultures entered the cell cycle synchronously to reach G_1/S-phase transition after 18–24 h of seeding and to complete the cell division cycle after 36–40 h (Fig. 1a). Cell cycle reinitiation in the presence of 0.1 μmol/l MTX, which is a dose that provokes 99% cell death in the HT-29 cell line [6], also allowed S-phase entrance, but cell cycle progression was dramatically delayed, so that the cells remained at S-phase during the following days (Fig. 1b and c). The earliest mitotic figures were observed from 3 days after cell cycle reinitiation and extension of the bromodeoxyuridine (BrdU) pulse–chase experiment shown in Fig. 1(c) revealed that the first wave of G_1 cells that had completed the cell cycle (i.e. labelled with BrdU) was not detected until the fifth day approximately (data not shown). Interestingly, increasing fractions of cells with a sub-G_1 DNA content and with more than 4*n* DNA content were observed at the latest treatment time points, suggesting that both apoptosis and mitotic alterations, respectively, occurred. To correlate these cell cycle effects with the cytotoxicity induced by MTX, we analysed the rates of cell death and survival during treatment by means of cell clonogenic assays. We observed a significant reduction of cell clonogenicity after 48 h of reinitiation in the presence of MTX, which was further reduced to a nadir of less than 1% of cell survival after 4 days during continuous treatment (Fig. 1d). Afterwards, increased cell clonogenicity was observed corresponding to an adaptive drug resistance phenomenon described previously and by which the surviving cell population adapts to grow at a low cell density in the presence of MTX [6]. Therefore, the cell population treated with a lethal dose of MTX could progress through the cell cycle, but most of the cells did so in an aberrant manner because progression was importantly slowed and cell viability mostly lost in the S phase. Importantly, a minor fraction of the cell population survived this aberrant cell cycle to undergo the above-mentioned adaptive resistance (see below).

As MTX has clastogenic effects [1] and it enhances centrosome reduplication [7], we postulated that aberrant cell cycle progression by MTX occurred in the presence of chromosome abnormalities. Consistent with this notion, we detected histone H2AX phosphorylation at serine-139, referred to as γ-H2AX, which is a marker of DNA double-strand breaks [19], as early as 48 h of cell cycle progression in the presence of MTX (Fig. 2). Therefore, MTX induces DNA damage in correlation with the loss of cell viability observed in the clonogenic assays (Fig. 1d). Moreover, figures of mitotic cells with multiple spindles, and spindle-unattached and condensed chromatin were frequently observed during MTX treatment, suggesting the induction of mitotic catastrophe [7]. This latter result raises an issue that merits further discussion. First, cells progressed slowly through the S phase from day 2 to day 4 and cell death (revealed by cell detachment and appearance of sub-G_1 cells) was not observed until days 4–5, coinciding with the major detection of mitosis (Fig. 1d). Second, sub-G_1 cells had undergone DNA replication as observed by BrdU pulse–chase labelling experiments (data not shown). Third, 80% of mitosis under treatment displayed aberrations, such as multiple spindle formation and nonaligned metaphase chromosomes [7]. Finally, micronucleated cells, a hallmark consequence of mitotic catastrophe [20], were typically observed as a result of MTX treatment (data not shown). Therefore, these results suggested that cells carrying DNA damage, or at least part of them, could progress to mitosis in the presence of MTX, so that one of the main consequences of treatment was the mitotic catastrophe of cycling cells carrying DNA damage. In addition to mitotic catastrophe, however, the concomitant occurrence of apoptosis or other cytotoxic effects compromising cell viability cannot be ruled out. Interestingly, the possibility that cells carrying DNA damage could enter mitosis would imply that these cells could bypass the G_2/M checkpoint in response to S-phase damage. Ascertaining this hypothesis might uncover a defect of HT-29 cells in the control of mitosis entrance in the presence of cell cycle abnormalities, such as damaged DNA or centrosome amplification. This phenomenon would be consistent with the fact that the G_2 DNA damage checkpoint has also been shown to be under p53 control in response to γ-irradiation or drugs [21,22].

We next asked whether the DNA damage checkpoint was induced in response to treatment. For this purpose, we analysed the phosphorylation status of Chk1, which is an effector kinase of the DNA damage checkpoint in response to UV or hydroxyurea treatments. Chk1 is

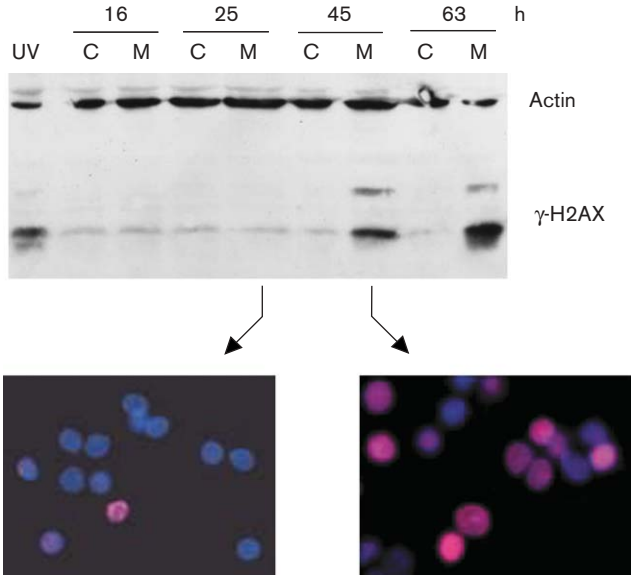
Fig. 1



Methotrexate (MTX) treatment delays S-phase entry and cell cycle progression concomitant with drug-induced loss of cell viability. (a) Nontreated HT-29 cells were arrested in G_0 and reinitiated by reseeding at subconfluence density (2×10^4 cells/cm^2). Cell suspensions were obtained at the indicated time points after reinitiation and stained with propidium iodide for flow cytometry analysis. The kinetics of cell cycle progression are shown by the percentages of cells with G_0/G_1 (open squares), S (open triangles) and G_2/M (closed triangles) DNA content profiles in each time point. (b) DNA content profiles by flow cytometry, as in (a), on synchronized cell cultures treated continuously with 0.1 μmol/l MTX at the time of reinitiation. Treated cells first entered S phase at 24 h after reinitiation, and S phase progression to G2/M phases occurred slowly through the following days. (c) Reinitiated cells as in (a) and (b) were labelled with bromodeoxyuridine (BrdU) for 1 h at day 2, and cell suspensions subjected to inmunofluorescence with fluorescein isothiocyanate (FITC)-labelled anti-BrdU antibodies (FITC log), stained with propidium iodide and analysed by flow cytometry. The BrdU labelling profile revealed that almost 100% of the cells were in the S phase after 2 days of MTX treatment. (d) Left: percentages of cells remaining attached in the culture plates during reinitiation in the presence of MTX; cell detachment was not detected until day 5, coinciding with mitosis progression as shown in (b), which is consistent with cell detachment driven by mitotic catastrophe. Right: percentages of cell viability during cell cycle reinitiation expressed as the percentage of colonies formed after seeding 10 cells/cm^2 from MTX-treated cultures with respect to 100% of clonogenicity from control cultures. Almost 90% of cell viability was lost by day 2 after reinitiation, a time point period coinciding with S-phase progression.

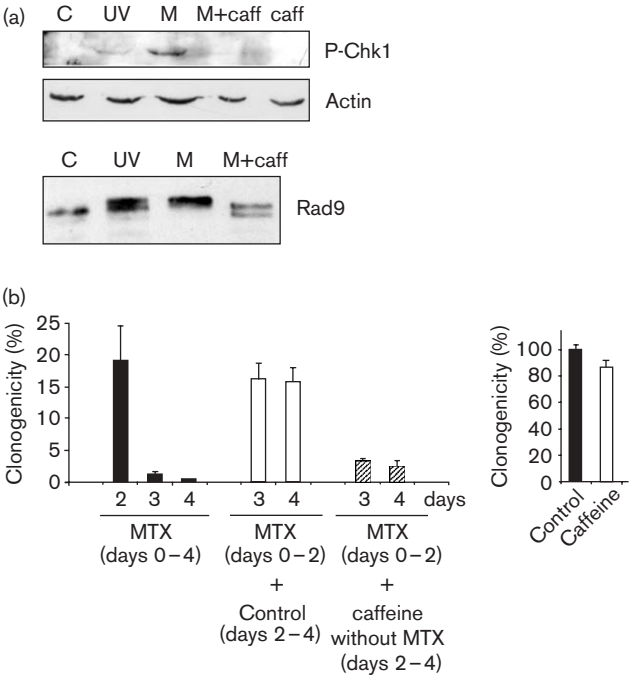
activated by the kinase activity of the checkpoint protein ATR and, when active, it directly phosphorylates key proteins involved in the cell cycle progression machinery, so that the cell cycle is delayed [23,24]. Using antibodies specific for the activated form of Chk1 (phosphorylated at serine-345) MTX-treated cells showed induction of

Fig. 2



Methotrexate (MTX) treatment induces DNA damage as cells progress through the S phase of the cell cycle. Reinitiated cells as in Fig. 1 were treated with MTX and cell extracts obtained at the indicated time points processed for Western Blot analysis. Briefly, harvested cells were lysed in denaturing buffer (6 mol/l urea, 50 mmol/l Tris–HCl, pH 6.8, 1% sodium dodecyl sulphate) and mild sonication (see Materials and methods), and 10 µg of whole-cell extracts were subjected to 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Mouse monoclonal anti-phospho-Histone H2AX (S139) antibody was used to detect DNA damage and anti-β-actin (clone AC-15; Sigma), used for normalization. H2AX phosphorylation took place first between 25 and 45 h after reinitiation in the presence of MTX (18.52 ± 6.74 and 73.13 ± 4.19% of γ-H2AX-positive cells at 25 and 45 h, respectively, expressed as the mean ± standard deviation from three independent experiments), which coincides with S-phase progression and loss of cell viability. HT-29 cells irradiated with UV (40 J/m^2) were used as controls of H2AX phosphorylation. C, nontreated control; UV, UV irradiation; M, MTX treatment. Bottom: two panels showing nuclei stained in red by immunofluorescence using anti-γ-H2AX at corresponding time points indicated by arrows; nuclei were counterstained with DAPI.

Fig. 3



Methotrexate (MTX)-induced DNA damage involves the activation of a caffeine-sensitive, Chk1/Rad9-mediated cell cycle checkpoint, which is required for cell survival upon drug withdrawal. (a) Western blot analysis using antibodies anti-phospho-Chk1 (P-Chk1) and rabbit polyclonal anti-human Rad9 (Rad9) on cell extracts from reinitiated cell cultures treated for 2 days. Chk1 and Rad9 phosphorylations were suppressed by the combined MTX plus 10 mmol/l caffeine treatment indicating its dependence on the caffeine-sensitive checkpoint. HT-29 cells irradiated with UV (40 J/m^2) were used as controls of Chk1 and Rad9 activation. Seventy-five micrograms of cell extract were loaded in each well. C, nontreated control; UV, UV irradiation; M, MTX treatment; M + caff, combined MTX plus caffeine treatment. (b) Left: percentages of cell clonogenicity as in Fig. 1 during cell cycle reinitiation by the continuous presence of MTX (closed bars), or by treatment with MTX during the first 2 days followed by MTX withdrawal and no treatment (open bars) or followed by MTX withdrawal and caffeine treatment (cross-hatched bars). The cell viability remaining after 2 days of MTX treatment is preserved but not increased by MTX withdrawal during the following 2 days, suggesting a period of cell repair, but is suppressed when caffeine is added after withdrawal. Right: the effects of 48-h caffeine treatment in cells not treated with MTX results in negligible effects on cell viability as measured using the cell clonogenicity assay.

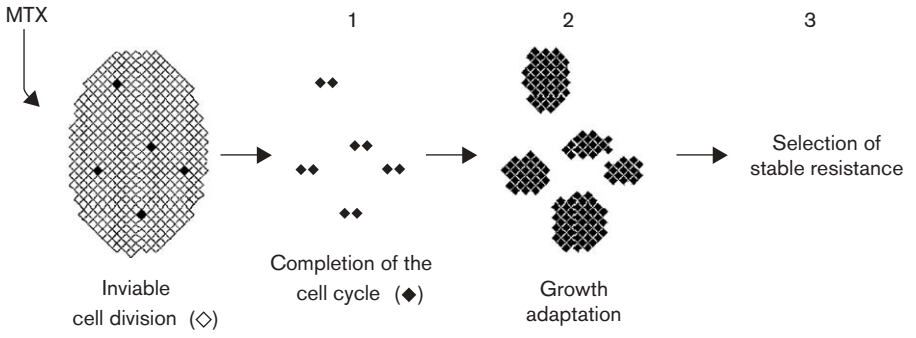
activated Chk1 to levels comparable to, or even higher than, UV-induced activation, while activated Chk1 remained undetectable in untreated cells (Fig. 3a). Moreover, we also analysed changes in Rad9, which forms part of the 9-1-1 complex, loaded to sites of DNA damage by the Rad17 complex [25]. We observed that phosphorylated forms of Rad9, similar to those induced by UV, were also induced under MTX treatment (Fig. 3a). As the 9-1-1 complex together with ATR are required for Chk1 activation in UV-treated cells [26], these results indicated that MTX induced a checkpoint response in HT-29 cells similar to the UV-induced ATR-mediated DNA damage checkpoint.

We have recently shown that resistance of HT-29 to low doses of MTX (0.1 µmol/l) is achieved through a multistep process that involves a stage of adaptive cell survival that takes place during the first week of treatment [6]. As this early phenomenon of cell survival coincides in time with the elongated cell cycle transition that follows MTX exposure, we hypothesized that the DNA damage checkpoint could promote DNA repair and cell survival during this early stage. To assess this hypothesis, we first analysed the cell clonogenicity remaining after 2 days of treatment and found that it was preserved for 2 additional days after drug withdrawal, without any increase in the cell number of cultures (Fig. 3b). This behaviour suggested that cells were recovering from MTX-induced cytotoxicity while the activated checkpoint was delaying S-phase progression. Afterwards, to ascertain whether cell

recovery in this stage depended on the checkpoint, we used caffeine as an inhibitor of ATM/ATR, upstream checkpoint kinases responsible for the activation of the DNA damage checkpoints in response to different insults [27]. Suppression of checkpoint activity by caffeine concomitant with MTX withdrawal dramatically suppressed the cell viability, whereas treatment with caffeine alone had no effects (Fig. 3b). Therefore, a caffeine-sensitive checkpoint was required for tumour cell recovery from MTX-induced abnormal cell cycle progression. Caffeine similarly suppressed Chk1, Rad9 and H2AX phosphorylations (Fig. 3a and data not shown), although we observed some γ-H2AX-positive cells with apoptotic morphology. As Chk1 is required for the repair of DNA double-strand breaks by homologous recombination [28], its inactivation by caffeine supports its role as a mediator of cell survival and recovery to MTX treatment. Similarly, suppression of Rad9 phosphorylation is consistent with the fact that it lies downstream of ATM/ATR [29]. Rad9 activation has also been described to protect from topoisomerase inhibitor-induced genotoxicity [11], thus supporting our notion that cell recovery from MTX-induced DNA damage is also mediated by the 9-1-1 complex. Finally, caffeine treatment after MTX withdrawal enhanced mitosis entry, but mitosis was almost exclusively composed of highly fragmented condensed DNA indicative of premature mitotic entrance in the presence of unrepaired double-strand DNA breaks (data not shown), as described in p53-deficient, aphidicolin-treated cells [30]. This result also indicates that the mechanism of checkpoint-mediated cell survival was the repair of MTX-induced double-strand DNA breaks. Nevertheless, and beyond the scope of the present report, only knockdown experiments against specific checkpoint proteins will reveal the particular pathway of cell cycle control involved in cell survival to MTX.

In the present work, we, therefore, report that the activation of a caffeine-sensitive checkpoint, with the characteristics of the ATR/Chk1-mediated checkpoint, is part of the cell response to MTX treatment in tumour cells. This is an issue that had not been addressed previously, despite MTX being a known genotoxic agent. More importantly, this checkpoint is presently shown to be required for a cell survival phenomenon to drug treatment that precedes the acquisition of stable resistance [6]. In particular, we describe that abnormal cell cycle progression by DNA damage is one of the main mechanisms of MTX cytotoxicity, and that, amid most of the cells that die by mitotic catastrophe or apoptosis, a small percentage of cells under treatment can escape from damage and complete the cell cycle in a viable manner. The caffeine-sensitive checkpoint appears to promote the viability of this cell cycle progression leading to the survival of some cells. These results will help to elaborate a mechanistic model to further understand the acquisition of stable resistance in tumour cell populations lacking resistant cells before treatment (Fig. 4). We have recently reported two main stages during resistance acquisition: (1) an initial stage of growth adaptation to continuous treatment in which the cell population is maintained at a low cell number, thus keeping available survival factors present in the culture medium [6], and (2) a later stage where stable resistance emerges in some of the surviving cell clones subjected to the selective pressure of treatment [7]. Regarding our present results, we propose that the DNA damage checkpoint is involved in an additional cell survival stage early after the

Fig. 4



Schematic model that illustrates the involvement of checkpoint-mediated cell survival in the acquisition of methotrexate (MTX) resistance. (1) HT-29 are sensitive to MTX and, early during treatment, most of the cells undergo massive cell death (white cells), but a minor fraction of cells are able to survive (black cells). The mechanism by which this minor fraction of cells survives to treatment does not involve the presence of a cell subpopulation already resistant before drug exposure and was unknown until now [6]. Our present results demonstrate that the cell death and cell survival processes during acute treatment occur within the first cell division cycle after the exposure to the drug, and that the DNA damage checkpoint is a major determinant of cell survival during the S-phase transition of this cycle. (2) Afterwards, surviving cells give rise to a cell population (black colonies) still sensitive to MTX, but able to grow at a low cell density in the continuous presence of the drug, as we previously reported [6]. This adaptation was shown to be due to the limited availability of extracellular nucleosides that, despite treatment, allow cell growth below a critical cell density. (3) Upon prolonged treatment, the phenomenon of cell sensitization and cell survival is repeated until some cells undergo permanent changes that lead to the emergence of clonal variants with stable resistance [7].

beginning of the treatment by allowing some cells to complete the first cell cycle after exposure to the drug. Therefore, inhibiting the DNA damage checkpoint may help prevent resistance to MTX by blocking one of the earliest stages of the resistance acquisition process, i.e. a stage that precedes the emergence of particular genetic changes that render cells drug stably resistant. Moreover, the phenomenon of cell survival to acute MTX treatment in a minor fraction of the HT-29 population resembles in-vivo situations in which tumour recurrence after drug treatment is often accompanied by drug resistance, e.g. minimal residual disease. Inhibiting the DNA damage checkpoint during MTX treatment may thus be a useful strategy to suppress tumour relapse by impeding the survival of residual cells. Finally, DNA damage induced by MTX and other antimetabolites occurs only in *TP53* mutant, G_1 checkpoint-defective tumour cells [1], so that it can be hypothesized that the effects of checkpoint inhibition on cell viability during MTX treatment will selectively target these tumour cells and conceivably spare G_1-arrested normal cells, thus providing an improved therapeutic index.

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References

1 Kinsella AR, Smith D, Pickard M. Resistance to chemotherapeutic antimetabolites: a function of salvage pathway involvement and cellular response to DNA damage. *Br J Cancer* 1997; **75**:935–945.
2 Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002; **108**:153–164.
3 Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology nd clinical use of methotrexate. *N Engl J Med* 1983; **309**:1094–1104.
4 Banerjee D, Mayer-Kuckuk P, Capiaux G, Budak-Alpdogan T, Gorlick R, Bertino JR. Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase. *Biochim Biophys Acta* 2002; **1587**:164–173.
5 Lesuffleur T, Barbat A, Dussaulx E, Zweibaum A. Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. *Cancer Res* 1990; **50**:6334–6343.
6 de Anta JM, Real FX, Mayol X. Low tumor cell density environment yields survival advantage of tumor cells exposed to MTX *in vitro*. *Biochim Biophys Acta* 2005; **1721**:98–106.
7 de Anta JM, Mayo C, Solé F, Salido M, Espinet B, Corzo C, *et al.* Methotrexate resistance *in vitro* is achieved by a dynamic selection process of tumor cell variants emerging during treatment. *Int J Cancer* 2006;**119**:1607–1615.
8 Cliby WA, Lewis KA, Lilly KK, Kaufmann SH. S phase and G_2 arrests induced by topoisomerase I poisons are dependent on ATR kinase function. *J Biol Chem* 2002; **277**:1599–1606.
9 Sampath D, Rao VA, Plunkett W. Mechanisms of apoptosis induction by nucleoside analogs. *Oncogene* 2003; **22**:9063–9074.
10 Flatten K, Dai NT, Vroman BT, Loegering D, Erlichman C, Karnitz LM, *et al.* The role of checkpoint kinase 1 in sensitivity to topoisomerase I poisons. *J Biol Chem* 2005; **280**:14349–14355.
11 Loegering D, Arlander SJ, Hackbarth J, Vroman BT, Roos-Mattjus P, Hopkins KM, *et al.* Rad9 protects cells from topoisomerase poison-induced cell death. *J Biol Chem* 2004; **279**:18641–18647.
12 Xiao Z, Xue J, Sowin TJ, Rosenberg SH, Zhang H. A novel mechanism of checkpoint abrogation conferred by Chk1 downregulation. *Oncogene* 2005; **24**:1403–1411.
13 Eastman A. Cell cycle checkpoints and their impact on anticancer therapeutic strategies. *J Cell Biochem* 2004; **91**:223–231.
14 Yin Y, Tainsky MA, Bischoff FZ, Strong LC, Wahl GM. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* 1992; **70**:937–948.
15 Linke SP, Clarkin KC, Di Leonardo A, Tsou A, Wahl GM. A reversible, p53-dependent G_0/G_1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Genes Dev* 1996; **10**:934–947.
16 Morales C, Ribas M, Aiza G, Peinado MA. Genetic determinants of methotrexate responsiveness and resistance in colon cancer cells. *Oncogene* 2005; **24**:6842–6847.
17 Toueille M, El-Andaloussi N, Frouin I, Freire R, Funk D, Shevelev I, *et al.* The human Rad9/Rad1/Hus1 damage sensor clamp interacts with DNA polymerase beta and increases its DNA substrate utilisation efficiency: implications for DNA repair. *Nucleic Acids Res* 2004; **32**:3316–3324.
18 Iacopetta B. TP53 mutation in colorectal cancer. *Hum Mutat* 2003; **21**:271–276.
19 Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998; **273**:5858–5868.
20 Roninson IB, Broude EV, Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Update* 2001; **4**:303–313.
21 Winters ZE, Ongkeko WM, Harris AL, Norbury CJ. p53 regulates Cdc2 independently of inhibitory phosphorylation to reinforce radiation-induced G_2 arrest in human cells. *Oncogene* 1998; **17**:673–684.
22 Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, *et al.* Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* 1999; **104**:263–269.
23 Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, *et al.* Chk1 is an essential kinase that is regulated by Atr and required for the G_2/M DNA damage checkpoint. *Genes Dev* 2000; **14**:1448–1459.
24 Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, *et al.* Aberrant cell cycle checkpoint function and early embryonic death in Chk1$^{-/-}$ mice. *Genes Dev* 2000; **14**:1439–1447.
25 Parrilla-Castellar ER, Arlander SJ, Karnitz L. Dial 9-1-1 for DNA damage: the Rad9–Hus1–Rad1 (9-1-1) clamp complex. *DNA Repair (Amsterdam)* 2004; **3**:1009–1014.
26 Weiss RS, Enoch T, Leder P. Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress. *Genes Dev* 2000; **14**:1886–1898.
27 Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, *et al.* Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res* 1999; **59**:4375–4382.
28 Sorensen CS, Hansen LT, Dziegielewski J, Syljuasen RG, Lundin C, Bartek J, *et al.* The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nat Cell Biol* 2005; **7**:195–201.
29 Chen MJ, Lin YT, Lieberman HB, Chen G, Lee EY. ATM-dependent phosphorylation of human Rad9 is required for ionizing radiation-induced checkpoint activation. *J Biol Chem* 2001; **276**:16580–16586.
30 Taylor WR, Agarwal ML, Agarwal A, Stacey DW, Stark GR. p53 inhibits entry into mitosis when DNA synthesis is blocked. *Oncogene* 1999; **18**:283–295.