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Title: 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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Manuscript Region of Origin:

Mels Sluyser
Editor
Anti-Cancer Drugs

Dear Dr. Sluyser,

Please find enclosed our revised version of the manuscript # ACD-06-176, originally entitled " The DNA damage checkpoint mediates residual tumour cell survival to methotrexate treatment as an initial step of acquired drug resistance ". I would like to thank the reviewers for their helpful suggestions and for poiting some concerns that, as addressed, I believe have help to improve the manuscript in much.

The reviewers' comments have been met point-by-point in the attached text.

I hope that we have answered all the reviewers' points of concern, and that the manuscript will be acceptable for publication.

Yours sincerely,

Xavier Mayol

Reviewer 1

The concept that Inhibition of Chk1 can potentiate cytotoxicity of antimetabolite, antimitotics and inhibitors of topoisomerases to prevent the immerging of resistance has been established in the field. Thus, this manuscript provides little new information.

Couple specific points:

Methotrexate is an antimetabolite drug, which blocks DNA synthesis and arrest cell in S phase irrespective to the p53 status in the first cell cycle, just like 5-FU. That is what author observed in Figure 1B. There are increasing amount of cells arrested in S phase (24-72 hrs). In p53 proficient cells, G1 checkpoint will be activated only upon DNA damaging which can be resulted a period time after treatment of MTX. The G1 checkpoint will effect on the cells leaking through S phase arrest by MTX. Conceptually, the author did not understand the difference-see the statement On pg.8 (According to the mutated TP53 status of the HT-29 cell line, cell cycle reinitiation in the presence of 0.1 µM MTX - which is a dose that provokes 99% cell death in the HT-29 cell line (6) - bypassed the G1 nucleotide starvation checkpoint allowing G1 progression and S phase entrance).

The Author also made wrong interpretation from Figure 1 B "the first wave of G1 cells that had completed the cell cycle were not detected until the 5th day approximately (Figure 1B and C)"(Pg.8). On Figure 1B, when HT29 cells treated with MTX for 120 hours, the majority cells were dead indicated by the subG0/G1 peak. Thus, from this histogram one cannot draw conclusion that cells are in G1. These could be the apoptotic cells with heterogeneous content of DNA. That is why you see such broad peaks spreading on the left of G2/M peak.

The reviewer is right when pointing that it has been already reported that inhibiting checkpoint proteins increases the efficacy of antineoplastic drugs, particularly DNA damaging drugs. We already stated this concept in the Introduction section of the mansucript (references 8-12). On the other hand, we would like to emphasize that our results go beyond the mere fact that the checkpoint counteracts the genotoxic effects of drugs, but we additionally propose that the checkpoint mediates a process of cell growth adaptation to drug treatment that precedes the acquisition of stable resistance, a phenomenon previously observed by us (de Anta et al., 2005 and 2006) and for which a mechanistic basis is now presented.

Point 1

Our statement was based on the fact that DNA damage by antimetabolite drugs, as well as the consequent ability to exert gene amplification, is dependent on the p53 status. We made a wrong interpretation of this fact when extending this notion to the process of entrance into S phase by methotrexate-treated cells, which – as the reviewer rightly points – is in fact independent of p53. Indeed, we have confirmed no differences in S phase entrance by BrdU labelling in methotrexate-treated p53+/+ and p53 -/- HCT116 colon cancer cells. This issue was studied in detail by the group of G. Wahl (Linke et al., Genes and Development, 1996) and we already cited this work in the original manuscript although our

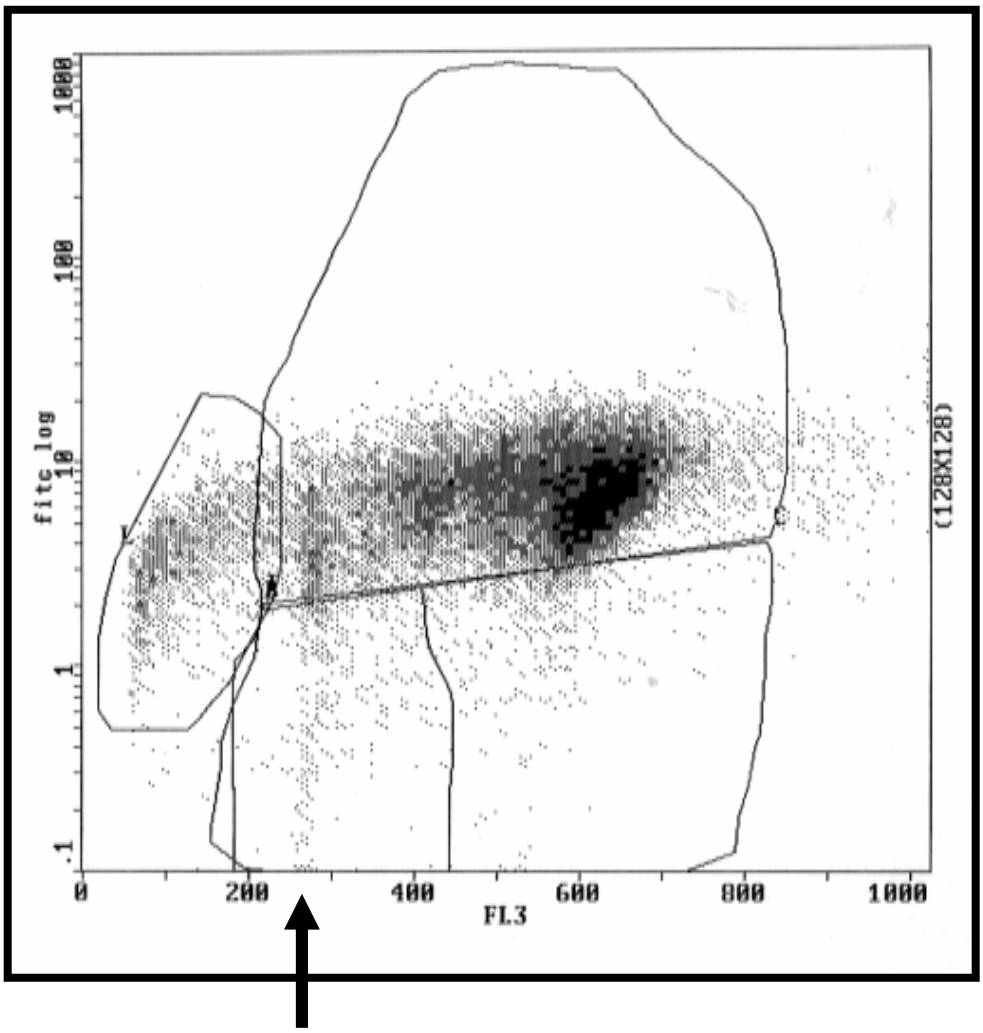
wrong statement. According to the reviewer, we have thus corrected the wrong statement. Nevertheless, our results showed that most of the HT-29 cell population progresses slowly through the S phase during the days of methotrexate treatment, which was not noted in the fibroblast cultures used by Linke et al., and appears to be the setting in which methotrexate-induced DNA damage takes place.

Point 2

We agree with the reviewer that, in the presence of MTX, cell cycle completion and return to G1 at the fifth day of cell cycle re-initiation can not be directly inferred from the figures shown. We should have been more precise when describing our results. Our conclusion was mainly based on the BrdU pulse-chase experiments partly shown in figure 1C. In these experiments, we also analyzed longer chase time points, i.e. 5 days, in which a faint peak of BrdU-labelled cells with G1 DNA content could be distinguished amid the cells of the sub-G1 population. The corresponding figure is included for reviewing purposes (figure to reviewer 1 comment 3). The result suggests that the minor fraction of surviving cells observed in the clonogenic assays corresponds to this BrdU-labelled G1 population. Since the statement in the original version of the manuscript, as such, led to confusion, we have rewritten the sentence mentioning this experiment.

We also agree with the reviewer that the main effect observed from figure 1B at the fifth day of treatment is the broad subG1 peak. In fact, we mentioned this result and suggested the occurrence of apoptosis in the sentence following the "G1 statement" critized by the reviewer.

Figure to reviewer 1 comment 3



Cells were re-initiated in the cell cycle, treated with MTX, and pulse-labelled and chased as in Figure 1C of the manuscript. Briefly, after 1-hour-pulse with BrdU at the second day of treatment (a time point in which >90% of the cells uptook BrdU, see Figure 1C of the manuscript), BrdU was withdrawn by changing the culture medium and cells were allowed to progress through the cell cycle until the fifth day when they were harvested. A faint BrdU-positive peak of cells with G1 DNA content (arrow) can be observed amid the cells with sub-G1 DNA content indicating that a minor fraction of the cells could escape the S phase arrest and complete the cell cycle.

Reviewer 2

This is an interesting manuscript in which Anta et al characterize DNA damage in HT-29 cells treated with methotrexate. Novel findings include the demonstration of H2AX phosphorylation, Chk1 phosphorylation, and Rad9 phosphorylation in these cells. I recommend the following minor revisions:

1. Several grammatical and spelling errors are spread throughout the manuscript:

a. page 4, 8th line: should be "Of note" since Noteworthily is not a word.

b. page 5, 9th line: "Despite MTX" should be changed to "Although MTX"

c. page 6, 3rd line and last line: methotrexate and methanol are mispelled

d. page 9, 7th line: "majoritarily" is not a word

e. page 12, 15th line: "were" should be changed to "where" or "in which"

f. page 13, 2nd line and last line: "recidivation" is not a word; "therapeutic" is mispelled

g. The affliations of the authors should be given by a,b,c..., not by 1,2,3.... The references in the text should be between square brackets. Example [1,3-5]. In the references list the dots after the numbers should be removed. Example: 1. should be 1

2. On page 9, the authors refer to mitotic catastrophe by MTX although they have no data to substantiate this. In order to accurately state that "...these results indicate that MTX-induced cell death took place by the mitotic catastrophe of cycling cells carrying DNA damage," the authors need to show evidence of mitotic catastrophe. Since this is not present in the ms, the statement should be removed.

3. In the 3rd or 4rth paragraph of the Introduction, the authors should add a reference to Morales et al; Oncogene; 2005 Oct 13;24(45):6842-7.

4. The Materials and Methods section should include the city and state of the companies from which reagants were purchased.

Point 1

All typing and spelling errors indicated by the reviewer have been corrected, as well as reference format.

Point 2

Our conclusion that mitotic catastrophe appears as a major mechanism of HT-29 cell death during MTX treatment was based on several observations that have now been listed in the text of the manuscript. Some of these results are going to be published in another manuscript (*International Journal of Cancer*, in press, available online at: http://www3.interscience.wiley.com/cgi-bin/abstract/112604627/ABSTRACT), and now we refer to that article in the manuscript. Following the reviewer's criticism, we have also changed our conclusion to just propose mitotic catastrophe as one likely mechanism of cell death by MTX, but that other mechanisms, such as apoptosis, can also occur.

Point 3

The reference required by the reviewer has been added in paragraph 4th of the Introduction, together with a sentence introducing it.

Point 4

The City and State of companies mentioned in the Materials and Methods section have been included.

Reviewer 3

In the presented paper Anta et al. provide data that the DNA damage checkpoint provide residual tumor cell survival to methotrexate treatment. First they showed that methotrexate treatment delayed the entry into S-phase and cell cycle progression. This effect was observed together with the methotrexate induced loss of cell viability. Next they observed after methotrexate treatment DNA damage measured as the diagnostic phosphorylation of H2AX. Moreover, the methotrexate induced DNA damages appears to involve a caffeine sensitive and Chk1/Rad9 mediated cell cycle checkpoint. These data suggested that this is required for the cell survival when methotrexate was withdrawn. The manuscript is clearly written and the authors provide for the first time a mechanistic investigation on of acquired resistance to drugs in cancer cells. The data are clear and the manuscript and in what follows are some suggestions to improve and clarify the paper.

Major point: Although the authors show the phosphorylation of checkpoint proteins and the inhibitory effect of caffeine on the phenomenon leading to acquired resistance, the assumption made in the title and in the text (p 11, line 2 and 3rd line before bottom) that the "DNA damage checkpoint mediates" the acquired resistance is too speculative in view of the data presented in this manuscript. The link between acquired resistance and DNA damage checkpoint is clearly shown, however, only knock-down experiments may prove a direct triggering of the acquired resistance by the checkpoint pathway. Considering this, although knock-down experiments are not required here, the authors should remain careful regarding the terms used to indicate the link between checkpoint and acquired resistance.

Minor points:

1. Preparation of the extract: What does "mild" sonication mean? Please give numbers.

2. The meaning of the indications "C, M and UV" should be indicated in the figure legend. Morover, no amounts of extracts are given. The control actin varies more than 50%. The authors should provide a quantification of the cells containing _H2AX foci at least at 25 and 45 hours in both control and treated cells.

3. Figure 3: For both panels a control of the effect of caffeine on both the controls cells and the UV treated cells is required. In addition, loading controls such as in Figure 2 should be included in this figure. Figure 3A: Here again no data are presented how much extracts were used. Moreover, an internal protein control like in Figure 2 is missing. Figure 3A again: Please explain more the right versus the left part of the figure.

4. Figure 4: can be omitted, since it is discussed in the text. With so far limited amount of data this model is not yet appropriate. It is sufficient to discuss it in the text.

5. Finally, several typing mistakes are present all over the text and in the figure legends, as well as in Figure 4. The paper should be checked by a native English speaker before resubmission.

Major point

The title has been revised according to the reviewer's suggestion. Moreover, a new sentence has been added at the end of the paragraph beginning in page 11 to include the reviewer's concern on the use of knock down experiments.

Point 1

The details of the sonication procedure used have been added in the Materials and Methods section.

Point 2

The meaning of the indications C, UV, and M, and the amount of extracts used in the western blot (10 μg) have been added in the legend of figure 2.

The percentages of cells stained with the γ-H2AX antibody after 25 and 45 hours of treatment with methotrexate have been calculated from three independent experiments and the resulting data have been included in the figure legend.

Point 3

The effects of caffeine on checkpoint activation and cell viability in the absence of MTX treatment have been included in Figure 3, and the manuscript text and figure legend have been modified accordingly.

We have removed the original western blot in figure 3A and added new western blots that include the control with caffeine alone and the corresponding normalization with actin antibodies. The figure legend has also been modified accordingly. In the former figure 3A, the right part and the left part of the figure were analyzed using different blotting membranes. Now, all the lanes belong to a single membrane displayed in one panel.

Finally, the amount of cell extract used for western blotting (75 μg) has been included in the figure legend.

Point 4

The reviewer argues that the experimental data is not sufficient to substantiate the model presented in figure 4. However, figure 4 is a compilation of data including those from the present manuscript, results previously published, and others from our paper presently in press (references are included in the figure legend). We thus believe that it is worth keeping the figure in the manuscript as it will help to explain more clearly the process of cell survival and resistance to methotrexate, which is multifasic and appears to involve multiple mechanisms. To adapt this figure to the reviewer's concern, we have simplified the illustration to indicate only the step of the multifasic process where the mechanism of cell survival presented in the present manuscript contributes to resistance, and have made some changes in the text for clarification.

After these changes, we believe that the illustration and its explanation in the text basically emphasise the central point of this manuscript: early cell survival to methotrexate treatment (as the first step of resistance acquisition) is dependent on the completion of the first cell division cycle after drug administration and, in turn, cell cycle completion is accomplished by means of checkpoint activation. In this manner, no more assumptions are made in the model which we assume was the major concern of the reviewer. On the other hand, we will gladly remove the figure from the manuscript if the reviewer is still concerned with its suitability.

Point 5

The manuscript has been checked for grammatical and spelling errors.

Title: 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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Running title: *Checkpoint-mediated tumour cell survival to methotrexate*

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Abstract

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 G2 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 prior to 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.

Keywords: *Methotrexate [D03.438.733.631.192.500]; Drug Resistance, Neoplasm [G12.392.395]; Tumour Cells, Cultured [A11.251.210.770]; Cell Survival [G04.335.316]; Cell Cycle [G04.335.134]; CHK1 protein,*

human [EC 2.7.1.37]; RAD9 homolog A (S. pombe) protein, human [139691-42-2]; Caffeine [D03.438.759.758.824.175].

Introduction

The process of tumour drug resistance is responsible for many therapeutic failures in the treatment of cancer and may be considered among the main consequences of tumour progression to increased malignancy. There are two types of drug resistance 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-deffective phenotypes [2]. Similarly, certain extracellular conditions may favour drug insensitation as inherent resistance, for example the presence of extracellular nucleosides that salvage nucleotide synthesis in antimetabolite-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 are responsible for the failure of some MTX-based therapies. On the other hand, colon cancer cell lines display *in vitro* high sensitivity 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 methotrexate 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 coinciding with the peak of MTX-induced cell death [6]. There are no resistant subpopulations 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]. Secondarily to 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 off 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 synthessis, such as MTX, result in the arrest at the G1/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 are known to arise on particular altered checkpoints [16]. Since 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 x 10^4 cells/cm^2 and cultured using DMEM medium, supplemented with 10% fetal bovine serum, at 37ºC with 7.5% CO_2. Where indicated, 0.1 μM methotrexate (Almirall, Barcelona, Spain), 10 mM caffeine (Sigma, St. Louis, MO), or both, were added to the cells. Cells were first synchronized by maintaining cultures at confluence for 6 days, so that inducing G0 arrest, and then they were released by re-seeding at 2 x 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 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 (6M urea, 50 mM tris-HCl pH 6.8, 1% SDS) by mild sonication on ice for 5 seconds using a UP100H ultrasonic procesor adjusted at 70% of amplitude (Hielscher Ultrasound Technology, Germany). Whole cell extracts were subjected to electrophoresis in 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Mouse monoclonal anti-phospho-Histone H2AX (S139) antibody clone JBW301 (Upstate, Charlottesville, VA), anti-beta-actin clone AC-15 (Sigma, St. Louis, MO), and anti-phospho-Chk1 (Ser345) (Cell signaling, Danvers, MA) were used following the manufacturer 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, IL).

Immunofluorescence assay. Cells growing on coverslips were fixed by immersion in 50% methanol – 50% acetone for 2 minutes. Then, they were permeabilized using 0.5% NP-40 in PBS for 10 minutes and blocked using 0.5% BSA in PBS for 20 minutes. Mouse monoclonal anti-phospho-Histone H2AX (S139) antibody was diluted 1:500 in 0.5% BSA and incubated for 1h at room temperature. After a brief wash with PBS, samples were incubated with secondary rhodamine-conjugated goat anti-mouse antibodies (Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature. Before visualization under the microscope, Vectashield mounting medium for fluorescence with DAPI stain was used (Vector Laboratories, Burlingame, CA).

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]. Since 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 G0 by maintaining cultures in a confluent state for one week, approximately, and released them from the arrest by seeding at subclonfluent density. Using this protocol, HT-29 cultures entered the cell cycle synchronously to reach G1/S phase transition after 18-24 hours of seeding and to complete the cell division cycle after 36-40 hours (Figure 1A). Cell cycle reinitiation in the presence of 0.1 μM 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 (Figure 1B and C). The earliest mitotic figures were observed from 3 days on after cell cycle re-initiation, and extension of the BrdU pulse-chase experiment shown in Figure 1C revealed that the first wave of G1 cells that had completed the cell cycle (i.e., labelled with BrdU) were not detected until the 5th day, approximately (data not shown). Interestingly, increasing fractions of cells with a sub-G1 DNA content and with more than 4n 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 analyzed 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 hours of re-

initiation in the presence of MTX, which was further reduced to a nadir of less than 1% of cell survival after four days during continuous treatment (Figure 1D). Afterwards, increased cell clonogenicity was observed corresponding to an adaptive drug resistance phenomenon described previously, 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 to this aberrant cell cycle to undergo the above mentioned adaptive resistance (see below).

Since 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 hours of cell cycle progression in the presence of MTX (Figure 2). Therefore, MTX induces DNA damage in correlation with the loss of cell viability observed in the clonogenic assays (Figure 1D). Moreover, figures of mitotic cells with multiple spindles and spindle-unatached, 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-G1 cells) was not observed until days 4-5 coinciding with the major detection of mitosis (Figure 1D). Second, sub-G1 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 non-aligned metaphase chromosomes [7]. Finally, micronucleated cells – a hallmark consequence of mitotic catstrophe [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. However, in addition to mitotic catastrophe, the concomitant occurrence of apoptosis or other cytotoxic effects compromising cell viability can not be ruled out. Interestingly, the possibility that cells carrying DNA damage could enter mitosis would imply that these cells could bypass the G2/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 G2 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 analyzed the phosphorylation status of Chk1, which is an effector kinase of the DNA damage checkpoint in response to UV or hydroxyurea treatments. Chk1 is 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, thus delaying cell cycle progression [23,24]. Using antibodies specific for the activated form of Chk1, phosphorylated at Ser-345, MTX-

treated cells showed induction of activated Chk1 to levels comparable to, or even higher than, UV-induced activation while activated Chk1 remained undetectable in untreated cells (Figure 3A). Moreover, we also analyzed 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 (Figure 3A). Since 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 μM) is achieved through a multistep process that involves an stage of adaptive cell survival that takes place during the first week of treatment [6]. Since 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 analyzed the cell clonogenicity remaining after two days of treatment and found that it was preserved for two additional days following drug withdrawal, without any increase in the cell number of cultures (Figure 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 while treatment with caffeine alone had no effects (Figure 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 (Figure 3A, and data not shown) although we observed some γ-H2AX positive cells with apoptotic morphology. Since 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 were 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 it has also been 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 knock down 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 is 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 scape 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 together will help to ellaborate a mechanistic model to further understand the acquisition of stable resistance in tumour cell populations lacking resistant cells before treatment (Figure 4). We have recently reported two main stages during resistance acquisition: 1) an initial stage of growth adaptation to continuous treatment where 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 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 to 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 resistant stably. Moreover, the phenomenon of cell survival to acute MTX treatment in a minor fraction of the HT-29 population resembles *in vivo* situations where tumour recurrence after drug treatment is often accompanied by drug resistance, for instance minimal residual disease. Inhibiting the DNA damage

checkpoint during MTX treatment may thus be a useful strategy to supress tumour relapse by impeding the survival of residual cells. Finally, DNA damage induced by MTX and other antimetabolites occurs only in *TP53* mutant, G1 checkpoint-defective tumour cells [1], so that it can be hypothesised that the effects of checkpoint inhibition on cell viability during MTX treatment will selectively target these tumour cells and conceivably spare G1-arrested normal cells, thus providing an improved therapeutic index.

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Figure legends

Fig. 1. Methotrexate treatment delays S phase entry and cell cycle progression concomitant with drug-induced loss of cell viability. **A**, Non-treated HT-29 cells were arrested in G0 and reinitiated by re-seeding at subconfluence density (2 x 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 G0/G1 (*open squares*), S (*open triangles*) and G2/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 μM MTX at the time of re-initiation. Treated cells first entered S phase at 24 hours 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 labeled with BrdU for 1 hour at day 2, and cell suspensions subjected to inmunofluorescence with FITC-labeled anti-BrdU antibodies (*FITC log*), stained with propidium iodide (*Propidium Iodide*), and analyzed by flow cytometry. The BrdU labeling 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 dettachment was not detected until day 5, coinciding with mitosis progression as shown in B, which is consistent with cell dettachment 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 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.

Fig. 2. Methotrexate treatment induces DNA damage as cells progress through the S phase of the cell cycle. Reinitiated cells as in Figure 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 (6M urea, 50 mM tris-HCl pH 6.8, 1% SDS) and mild sonication (see Materials and methods), and 10 μg of whole cell extracts were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Mouse monoclonal anti-phospho-Histone H2AX (S139) antibody was used to detect DNA damage and anti-beta-actin (clone AC-15, Sigma), used for normalization. H2AX phosphorylation took place first between 25 and 45 hours 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 hours, 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*, non-treated control; *UV*, UV irradiation; *M*, MTX treatment. *Below*, two panels showing nuclei stained in red by inmunofluorescence using anti-γ-H2AX at corresponding time points indicated by arrows; nuclei were counterstained with DAPI.

Fig. 3. Methotrexate-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 mM 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*, non-treated control; *UV*, UV irradiation; *M*, MTX treatment; *M+caff*, combined MTX plus caffeine treatment. **B**, left pannel, percentages of cell clonogenicity as in Figure 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 pannel, the effects of 48-hour caffeine treatment in cells not treated with MTX results in negligible effects on cell viability as measured using the cell clonogenicity assay.

Fig. 4. Schematic model that illustrates the involvement of checkpoint-mediated cell survival in the acquisition of 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 (ref. 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 methotrexate but able to grow at a low cell density in the continuous presence of the drug, as we previously reported (ref. 6). This adaptation was shown to be due to the limited the availability of

extracellular nucleosides that, despite treatment, allow cell growth below a critical cell density. **3**, upon prolongued 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 (ref. 7).

Figure1

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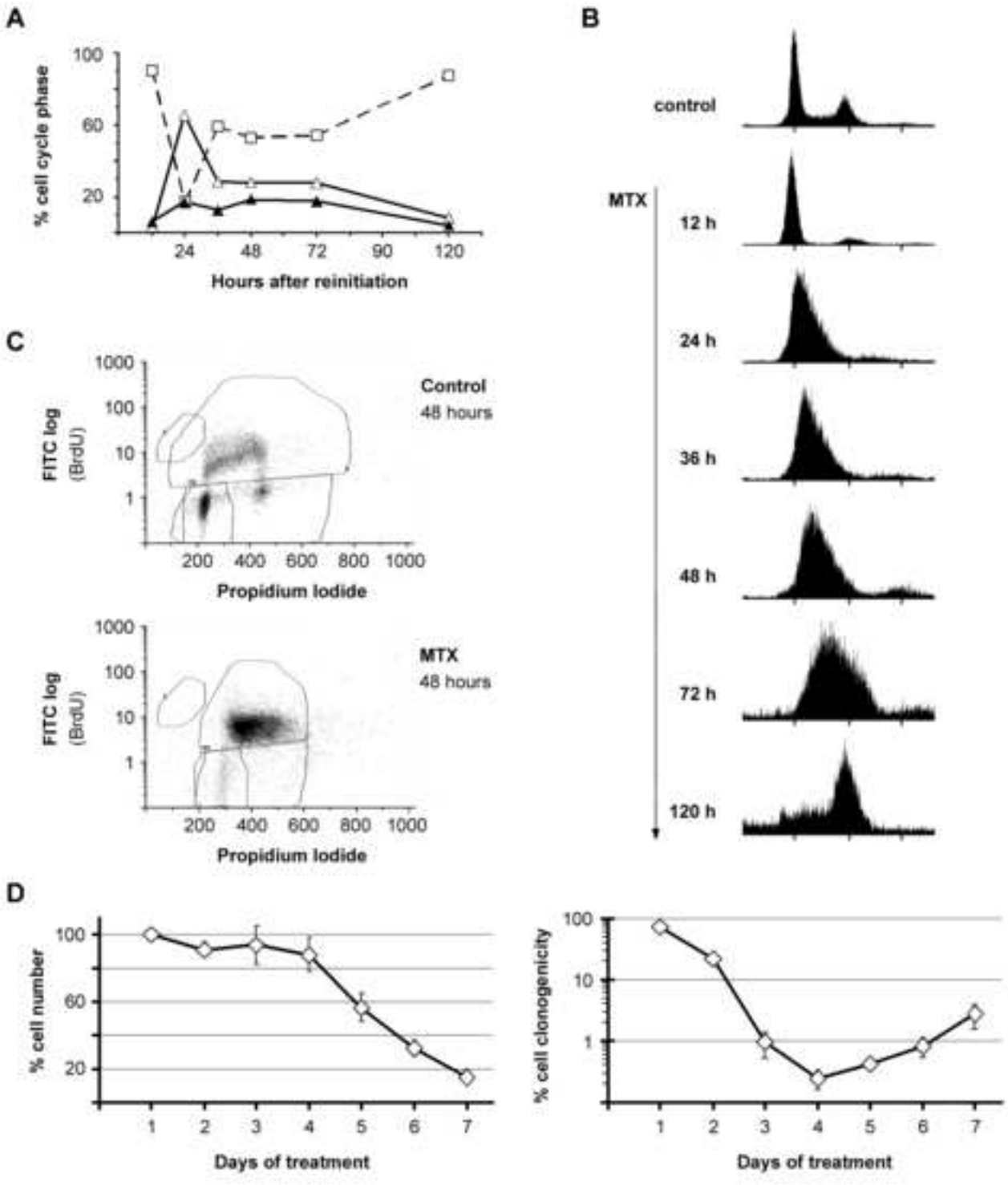

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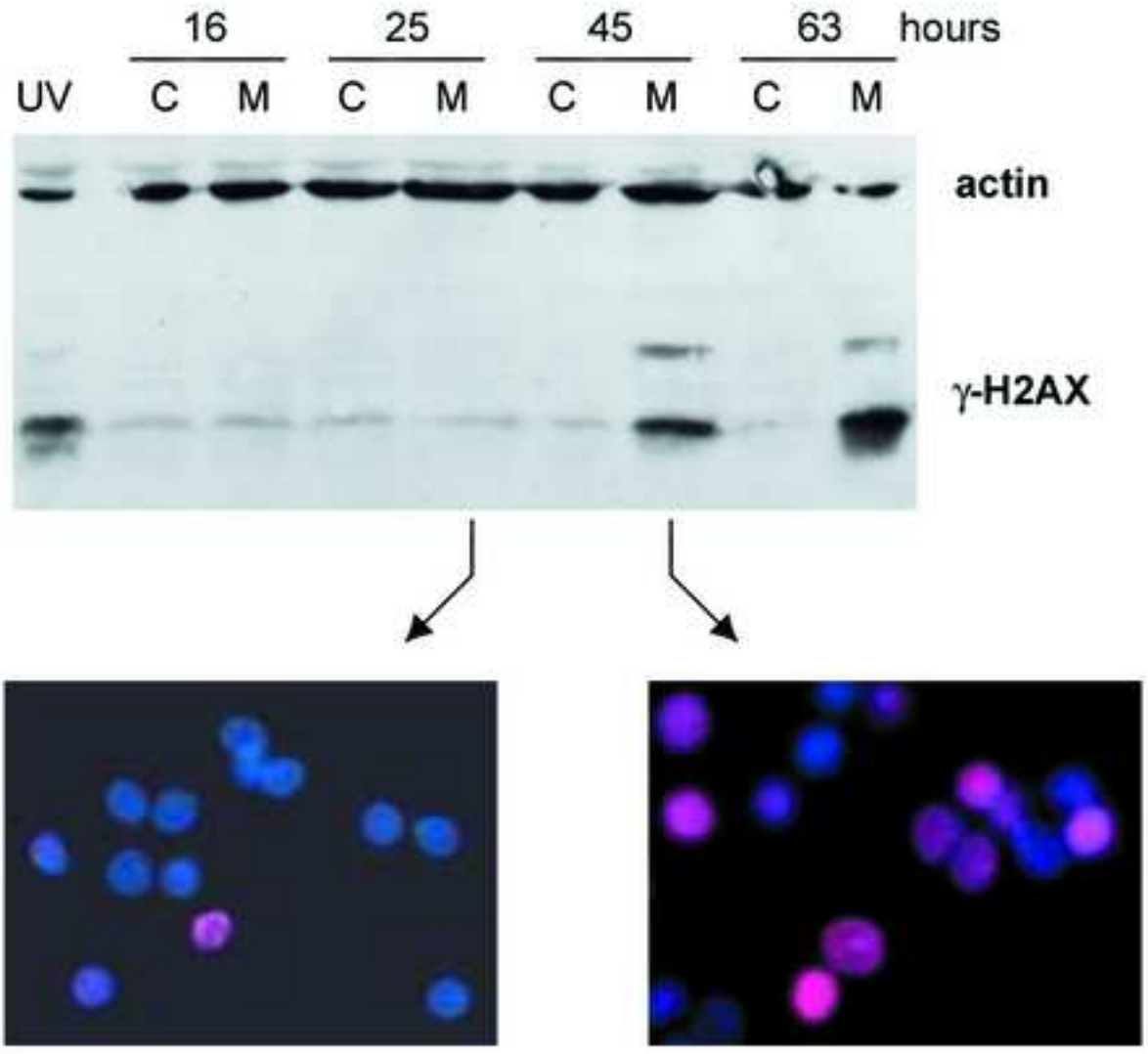

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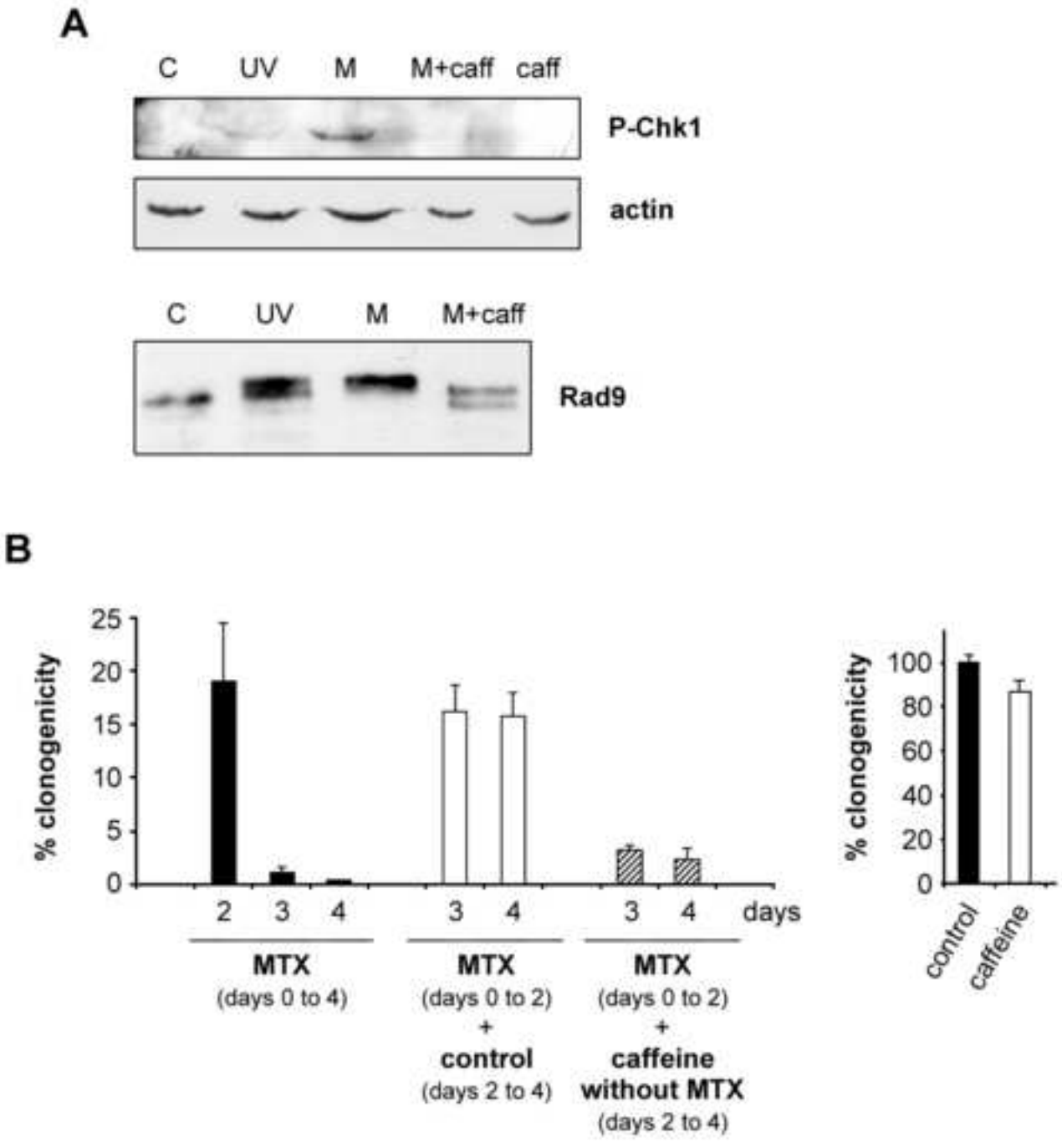

Figure4

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