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ORIGINAL ARTICLE

Caffeine-suppressed ATM pathway leads to decreased p53 phosphorylation and increased programmed cell death in gamma-irradiated leukaemic molt-4 cells

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Summary
Ionising radiation (IR) is one of the main treatment modalities in oncology. However, we still search for substances which can radio-sensitize tumour cells. In this study we used caffeine, a non-specific ataxia-telangiectasia mutated kinase (ATM) inhibitor, and studied its effect on the activation of the proteins involved in cell cycle control and the induction of apoptosis in human T-lymphocyte leukaemic MOLT-4 cells (p53 wt). We evaluated the expression of the tumour-suppressor p53 (itself and phosphorylated on Ser^{15} and Ser^{392}), the cell cycle regulator p21, and the anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1). After treatment with 2 mM caffeine, the cells were irradiated by 1 or 3 Gy, lysed and the proteins detected by Western-blotting. Apoptosis was determined by flow-cytometric annexin V/propidium iodine detection. Irradiation by 1 or 3 Gy induced p53 phosphorylation at Ser^{15} and Ser^{392} after 2 h with maximum after 4 h. Adding caffeine significantly inhibited Ser^{15} phosphorylation, which is ATM-dependent but surprisingly also Ser^{392} phosphorylation, which is ATM-independent, suggesting that caffeine might have another cellular target (protein kinase). Similarly, caffeine caused a substantial decrease in p21 in combination with both doses of IR and also Mcl-1 was down-regulated. Three days after irradiation, caffeine significantly increased induction of apoptosis. The ATM/p53 pathway was suppressed by caffeine, which led to increased apoptosis accompanied by a p53-independent decrease in Mcl-1. It also caused down-regulation of p21, which possibly contributed to the shortened cell cycle arrest necessary for effective DNA repair and thus impeded radio-resistance. Caffeine promotes the cytotoxic effect of ionising radiation and provides a possible platform for the development of new anti-cancer therapeutics known as radio-sensitizers.

Key words: ATM; p53; p21; Mcl-1; caffeine; ionising radiation; MOLT-4

Abbreviations: IR, ionising radiation; ATM, ataxia telangiectasia mutated kinase; ATR, ATM- and Rad3-related protein kinase; chk-2, checkpoint kinase-2

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INTRODUCTION

Exposure of mammalian cells to ionising radiation (IR) leads to DNA damage such as double strand breaks (DSB). A very early step in the DNA repair is rapid intermolecular autophosphorylation and activation of ataxia-telangiectasia mutated (ATM) kinase (Lavin et al. 1995, Bakkenist and Kastan 2003). The importance of ATM as a central controller

of cellular responses to DSB is undeniable; it regulates all three cell cycle checkpoints, DNA repair, and apoptosis (Khanna et al. 2001). ATM functions in the signalling pathways as a co-ordinator of the key components, since it regulates its substrates by phosphorylation. These are e.g. p53, murine-double minute protein (mdm2), and checkpoint kinase-2 (chk-2) managing the G1 checkpoint (Matsuoka et al. 2000, Maya et al. 2001) and many others for the transient S-phase arrest and the G2/M checkpoint.

IR is one of the main treatment modalities in oncology. However, it can generate a series of unwanted events inside the cells and therefore it is recommended that doses be minimised as much as possible. This might be achieved by the combination of irradiation with substances which are capable of sensitising the target cells towards radiation; radio-sensitisers.

In a number of cells, ATM and its regulatory function can be inhibited by many protein kinase inhibitors (Blasina et al. 1999, Kaufmann et al. 2003). In this work we focused on caffeine, which could act on cell cycle checkpoints and affect the progression of the cell cycle, but its impact on the apoptosis of tumour cells has not yet been properly identified. Steinmann et al. (1991) stated that caffeine is capable of abrogating cell cycle checkpoints in several different mammalian cell lines.

Caffeine is a methylxanthine possessing many different cellular effects. A number of *in vitro* and *in vivo* studies have demonstrated that caffeine modulates both innate and adaptive immune responses via inhibition of cAMP-phosphodiesterase; another group of effects induced by caffeine is mediated through its inhibitory action on adenosine receptors, which could have a direct impact on neovascularization of human tumours, and finally, some studies have proved that caffeine affects the anti-tumour activity of DNA-intercalating drugs (reviewed in Sabisz and Skladanowski 2008). The precise mechanism is still under debate, but some have shown that the radio-sensitising effects of caffeine are associated with the disruption of multiple DNA damage-responsive cell cycle checkpoints and DNA repair (e.g. Asaad et al. 2000, Deplanque et al. 2001, Block et al. 2004). Undoubtedly, the effects of caffeine are to a great extent cell-type dependent.

In this paper we describe the effect of caffeine on T-lymphocyte leukaemic MOLT-4 cells in the perspective of ATM inhibition and subsequent inhibition of its targets involved in DNA repair, cell cycle control, and apoptosis. We conclude that pre-treatment of MOLT-4 cells with caffeine prior to gamma-irradiation has a radio-sensitising effect.

MATERIALS AND METHODS

Cell culture and culture conditions

The human T-lymphocyte leukaemia MOLT-4 cells were obtained from the American Type Culture Collections (Manassas, USA). The cells were cultured in Iscove's modified Dulbecco's medium (Sigma, St. Louis, USA) supplemented with 20% fetal calf serum, 0.05% L-glutamine, 150 UI/ml penicillin, 50 μg/ml streptomycin in a humidified incubator at 37 °C and controlled 5% CO_2 atmosphere. The cultures were divided every second day by dilution to a concentration of 2×10^5 cells/ml. Cell lines in the maximal range of up to 20 passages were used for this study.

Gamma-irradiation

Exponentially growing cells were suspended at a concentration of 2×10^5/ml. Aliquots of 10 ml of cell suspension were plated into 25 cm^2 flasks (Nunc, Wiesbaden, Germany) and irradiated at room temperature using a ^{60}Co γ-ray source with a dose-rate of 0.4 Gy/min, at a distance of 1 m from the source. After irradiation, the flasks were placed in a 37 °C incubator with 5% CO_2 atmosphere and aliquots of the cells were removed at various times after irradiation by 1 or 3 Gy for further analysis. The cells were counted and cell viability was determined with the Trypan blue exclusion assay.

Caffeine exposure

We added 2 mM caffeine (Sigma) to the cells 1 h prior to irradiation.

Flow-cytometry

For apoptosis detection we used an Apoptest-FITC kit (Dako-Cytomation, Brno, Czech Republic). During apoptosis, cells expose phosphatidylserine at the cell surface. Annexin V (A) is a phospholipid binding protein, which (in the presence of calcium ions) binds selectively and with high affinity to phosphatidylserine. Cells with a permeable cell membrane (late apoptotic or necrotic) were detected by propidium iodide (PI) staining. Flow-cytometric analysis was performed on a Coulter Epics XL flow cytometer equipped with a 15mW argon-ion laser with excitation capabilities at 488 nm (Beckman Coulter, Fullerton, USA). A minimum of 10,000 cells was collected for each two-colour sample in a list mode file format. List mode data were analysed using Epics XL System II software (Beckman Coulter).

Electrophoresis and Western blotting

At various times after irradiation (2, 4, 16, and 24 h), the cells were washed with phosphate buffer saline and lysed. Whole cell extracts were prepared by lysis in 500 µl of lysis buffer (137 mM NaCl, 10% glycerol, 1% n-octyl-β-glucopyranoside, 50 mM NaF, 20 mM Tris, 1 mM Na_3VO_4, pH 8 – all from Sigma, St. Louis, USA – and 1 tablet of Complete™ Mini, Roche, Manheim, Germany). The lysates containing equal amount of protein (30 µg) were loaded onto a 12% SDS (sodium dodecyl sulphate) polyacrylamide gel. After electrophoresis, the proteins were transferred to a PVDF (polyvinylidene difluoride) membrane (BioRad, Hercules, USA), and hybridized with an appropriate antibody: anti-p53 and anti-p53 Ser^{392} (1:1000 and 1:500, Exbio, Prague, Czech Republic); anti-p53 Ser^{15} (1:1000, Calbiochem, San Diego, USA), Mcl-1, p21, and anti-β-actin (1:1000, 1:500 and 1:10000, Sigma). After washing, the blots were incubated with secondary peroxidase-conjugated antibody – 1:1000/1:10000 (Dako, High Wycombe, UK) and the signal was developed with an ECL detection kit (BM Chemiluminescence – POD, Roche, Manheim, Germany) by exposure to a film.

RESULTS AND DISCUSSION

Tumour cells exposed to caffeine tend to be more sensitive to IR and other genotoxic agents. In spite of the fact that the radio-sensitising effects of caffeine have been studied for over last two decades, the underlying mechanism is still in debate. Nevertheless, it is more than likely that this phenomenon is linked to signalling pathways critical for adequate recovery from irradiation, namely DNA damage checkpoints. Accumulating evidence indicates that under normal conditions the crucial role in checkpoint control is played by the members of phosphatidylinositol 3-kinase related kinases, especially by ATM (Hoekstra 1997, Pandita 2003). ATM derives from ataxia-telangiectasia (A-T), human autosomal recessive disorder, in which a gene is mutated. A-T cells display specific genotype and in addition to increased radio-sensitivity they suffer from genomic instability, cancer predispositions and increased sensitivity to inhibitors of topoisomerase (Lavin and Shiloh 1997). ATM integrates the cellular response to DSB caused by these agents via phosphorylation of the key proteins involved in cell cycle regulation, DNA repair, and apoptosis. This was studied in our previous work on γ-irradiated MOLT-4 cells, which proved ATM/chk-2/p53 signalling pathway to be functional (ATM, chk-2, p53, and mdm2 were phosphorylated rapidly after irradiation) and both of the p53 phosphorylations on Ser^{15} and Ser^{392} were detected after irradiation (Tichý et al. 2007). Moreover, Sarkaria et al. (1999) showed on lung adenocarcinoma cells that caffeine inhibits the catalytic activity of both ATM and ATM and Rad3-related kinase (ATR). Kaufmann et al. (2003) have stated that a caffeine concentration that inhibits ATM by 50% is 1mM and the 50% inhibitory concentration for ATR is about 3mM (*in vitro*). These concentrations define the range at which caffeine effectively reverses cell cycle checkpoints and enhances cytotoxicity in carcinogen-damaged cells without significant toxicity of its own. In this study we used a caffeine concentration of 2 mM. Lower concentrations are less effective at reversing checkpoint function and higher concentrations cause a reduction in DNA synthesis associated with cytotoxicity.

Phosphorylations of p53 are inhibited by caffeine

In this study, we focused on one of the typical molecules, whose activation is linked to IR – protein p53. The specific mechanisms that determine whether p53-dependent cell cycle arrest or p53-dependent apoptosis prevails in response to specific DNA damage are poorly understood. Sarkaria et al. (1999) reported that caffeine inhibits γ- and UV radiation-induced phosphorylation of p53 on Ser^{15}, a modification that may be directly mediated by ATM. However, Kaufmann et al. (2003) in their work on human fibroblasts described the ATM-dependent phosphorylation of p53 after γ-irradiation as resistant to caffeine. Importantly, in our present work, we examined the implication of caffeine on phosphorylations on Ser^{15} and Ser^{392} and we found them strongly affected by this agent. As a matter of fact, both of the phosphorylations were substantially inhibited throughout the whole period of the experiment.

P53 was present in the intact cells and its amount dramatically increased after irradiation in a dose-dependent manner. It was up-regulated 2 h after irradiation by doses of 1 and 3 Gy with maximum after 16 h. Caffeine caused a mild decrease in p53 in irradiated and partially even in non-irradiated cells (Fig. 1).

Phosphorylation on Ser^{15} is crucial for IR-induced DNA damage response. ATM, ATR, and DNA-dependent protein kinase are responsible for this phosphorylation, whereby p53 stability is maintained (Shieh et al. 1997, Tibbetts et al. 1999, Helt et al. 2005). The phosphorylation is important for activation of p53 after exposure to IR, since it impairs

the ability of mdm2 to inhibit p53-dependent transactivation (Shieh et al. 1997). Phosphorylation of p53 on Ser^{15} was detected only in irradiated cells after 2 h with a maximum after 4 h. Caffeine caused a substantial decrease in this post-translational modification after irradiation (Fig. 1).

Phosphorylation of p53 on Ser^{392} enhances its sequence-specific DNA binding (Criswell et al. 2003). Sakaguchi et al. (1997) showed that phosphorylation on Ser^{392} stabilizes the tetramer formation of p53 that is critical for its ability to activate transcription because it facilitates phosphorylation of the transactivation domain due to a more favourable conformation. Casein kinase 2 (CK2) phosphorylates p53 on Ser^{392}, but Claudio et al. (2006) demonstrated that Cdk9 itself is capable of such phosphorylation independently of CK2. Saito et al. (2002) proposed the N-terminal p53 phosphorylation sites to be grouped into two categories with respect to dependence on ATM for rapid phosphorylation in response to IR. One group, consisting of Ser^{6}, Ser^{33}, Ser^{315}, and Ser^{392}, is independent of ATM and constitutively phosphorylated at low levels; the other group, consisting of Ser^{9}, Ser^{15}, Ser^{20}, and Ser^{46}, is ATM-dependent, typically with a rapid response to IR-induced damage, presumably DSB. Notably, our data show that caffeine treatment suppressed Ser^{392} phosphorylation and as long as it is ATM-independent, caffeine may have another cellular target. We suggest that caffeine inhibits another protein kinase, which executes this phosphorylation.

Sarkaria et al. (1999) also stated that the radio-sensitizing effects of caffeine are related to the inhibition of ATM and ATR and that both proteins are relevant targets for the development of novel anti-cancer agents. After γ-irradiation the pivotal role is played by ATM, whereas ATR is indispensable after UV-radiation or cellular damage caused by intercalating cytostatic agents. Nevertheless, these data must be perceived in the light of *in vitro* studies, because other groups reported multiple ATM-ATR substrates including chk-1 and chk-2 to be hyper-phosphorylated in cells treated with caffeine and IR, and although caffeine is an inhibitor of ATM-ATR kinase activity *in vitro*, it can block checkpoints without inhibiting ATM-ATR *in vivo* (reviewed in Cortez, 2003).

Caffeine causes down-regulation of p21

Besides providing the evidence that caffeine inhibits ATM-dependent phosphorylation of p53 in γ-irradiated leukaemic cells we also report on the inhibition of p21. This protein functions as inhibitor of cyclin-dependent kinases and thus can stop the cell cycle (reviewed in Schwartz, 2002).

Protein p21 was not detected in non-irradiated or caffeine-treated cells. It was up-regulated 4 h after irradiation by doses of 1 and 3 Gy with maximum after 24 h, but this effect was markedly inhibited by caffeine (Fig. 1). This is in accordance with other studies describing caffeine-induced ATM-dependent inhibition of p21 (Hill et al. 2008, Meng and Jiang 2009). We observed a link between radio-resistance and p21 up-regulation also in the promyelocytic leukaemic cell line HL-60 (p53-negative). After 2 Gy we did not detect an increase in p21, but in the radio-resistant line HL-60-R (which received a fractionated dose of 60 Gy before the experiment), an increase in p21 was already obvious 4 h after irradiation (unpublished data).

Caffeine causes down-regulation of Mcl-1

We evaluated the downstream effect of caffeine and we checked the expression of the anti-apoptotic member of the Bcl-2 protein family, myeloid cell leukemia 1 (Mcl-1), which consists of the key regulators of cell death. Mcl-1 acts as an apical molecule in control of apoptosis and propagation of cell survival (Borner 2003). A decrease in Mcl-1 is associated with apoptosis induction, since it blocks IR-induced apoptosis and inhibits clonogenic cell death by maintaining mitochondrial integrity via interaction with pro-apoptotic partners (Borner 2003, Skvara et al. 2005). Also, a novel role for this protein has been explored; Jamil et al. (2005) concluded that Mcl-1 is essential in ATR-mediated chk-1 phosphorylation, perhaps acting as an adaptor protein. We detected the anti-apoptotic protein Mcl-1 in non-irradiated cells. Its amount in caffeine-treated cells did not change during the whole 24 h period of the experiment. Sixteen h after irradiation by the dose of 3 Gy we observed a partial decrease in Mcl-1. A very pronounced decrease in Mcl-1 was observed 24 h after irradiation. In our previous work on HL-60 we found that the amount of Mcl-1 initially increased after irradiation by a sublethal but not lethal dose and later (when apoptosis occurred) it decreased in a dose-dependent manner (Tichý et al. 2008). In the recent experiments we did not observe any up-regulation of Mcl-1, but this protein was markedly down-regulated 24 h after irradiation by a combination of gamma-radiation with caffeine. Moreover, this obvious decrease in Mcl-1 was p53-independent (Fig. 1).

Caffeine increases induction of apoptosis

The phase at which leukemia cells enter apoptosis depends on the nature of the insult. In the case of

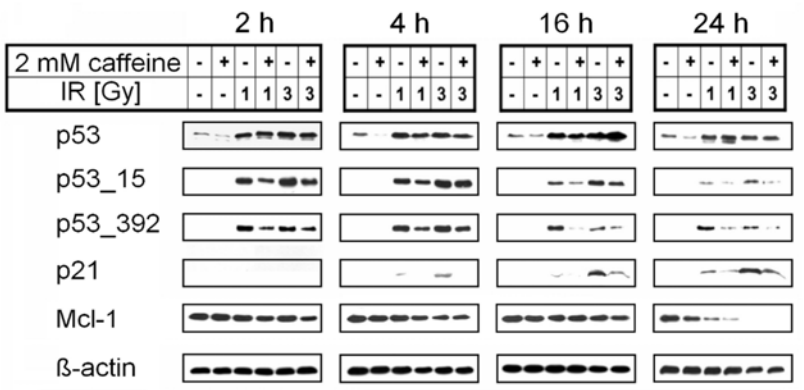

Fig. 1. **Inhibitory effect of caffeine on proteins involved in cell cycle regulation and apoptosis.** The MOLT-4 cells were irradiated and lysed as indicated; proteins were electrophoretically separated and detected by immunoblotting. Combination of caffeine (added 1 h prior irradiation) and ionising radiation caused substantial decrease in phosphorylation of p53 and down-regulation of p21 and Mcl-1. Representative Western blots are shown.

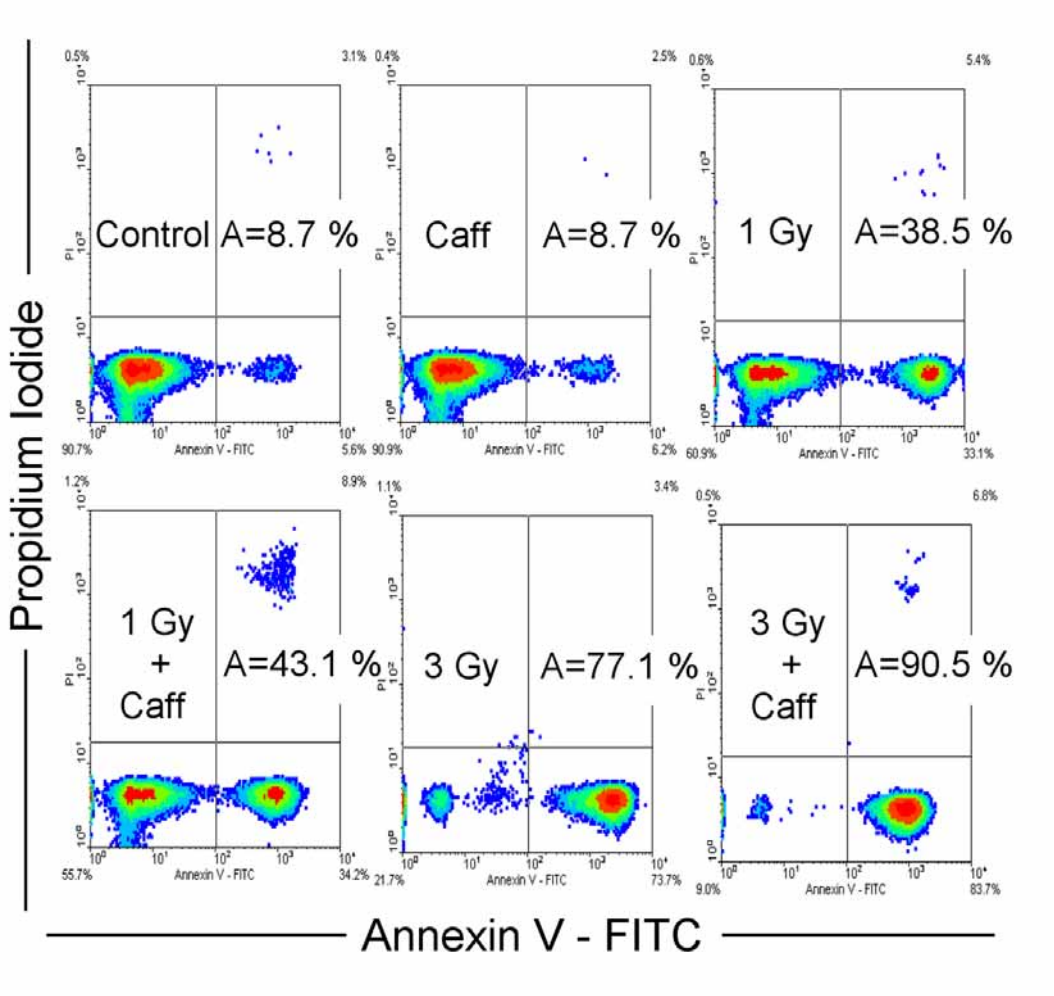

Fig. 2. **Effect of caffeine and ionising radiation on induction of apoptosis.** The MOLT-4 cells were treated with caffeine (added 1 h prior to irradiation and washed out 24 h post irradiation), irradiated, stained with annexin V/propidium iodide and then, apoptosis was measured by flow-cytometry 72 h after irradiation. Combination of caffeine and ionising radiation leads to increased apoptosis.

UV- or gamma-radiation it is G1-phase (Feng et al. 2007). This group also demonstrated on MOLT-4 cells that most, if not all, apoptosis is initiated in this specific cell cycle phase. Moreover, cells progressing through the cell cycle without stopping at checkpoints could avoid apoptosis. The apoptotic pattern and schedule varies with changes in the cell cycle and the uncoupling of apoptosis from cell cycle progression may lead to uncontrolled proliferation. Cellular proliferation is a manifestation of passage through the cell cycle, which is regulated at various checkpoints (Bartek and Lukas 2001). We evaluated induction of apoptosis 72 h after irradiation by annexin V/propidium iodide staining. The annexin V/propidium iodide staining revealed 8.7% of apoptotic cells in the control and the same amount (8.7%) in caffeine-treated cells. After irradiation by 1 or 3 Gy we found 38.5 or 77.1% of apoptotic cells, respectively. Adding caffeine induced an increase in the number of apoptotic cells to 43.1 or 90.5%, respectively (Fig. 2). It is likely that caffeine abrogated G2 cell cycle arrest, as previously confirmed by others (Kastan et al. 1991, Sarkaria et al. 1999, Vávrová et al. 2003) and therefore we observed a higher proliferation rate in caffeine- and IR-treated cells than in irradiated cells during the first days after irradiation (unpublished data). In our previous work on HL-60 cells, caffeine-induced apoptosis was observed in the late intervals after irradiation – 7 to 10 days (Vávrová et al. 2003). Linkage of radio-resistance with the time of DNA damage repair was also apparent in our experiments on cells irradiated by low dose-rate. When the cells were irradiated in the G2-phase, their radio-resistance was higher than after single irradiation. On the other hand, when the cells entered mitosis their radio-resistance decreased (Vávrová et al. 2004).

It seems that, DNA damage can result in the activation of cell cycle checkpoints and subsequent cell cycle arrest, which may lead either to DNA repair or in the case of extensive DNA lesions, to apoptosis. It is likely that caffeine effectively abrogates DNA damage G2/M checkpoint activation and could permit entry of arrested cells into M-phase by inhibiting ATM. And so we observed increased proliferation 24 h after irradiation and not apoptosis immediately after Mcl-1 decrease; nevertheless, later (72 h after irradiation), the cells with propagated unrepaired DNA died by increased apoptosis.

CONCLUSION

In this work, we show that caffeine suppresses ATM/p53 signalling pathway in human T-lymphocyte leukaemic MOLT-4 cells. P21 up-regulation was suppressed as a result of inhibited p53 phosphorylation on Ser^{15} and surprisingly also Ser^{392} phosphorylation, which is ATM-independent, suggesting that caffeine might have another cellular target (protein kinase). Notably, we observed a significant p53-independent decrease in anti-apoptotic Mcl-1 expression. These data suggest that caffeine increases the G2/M block override, diminishes repair of gamma-irradiation induced DNA damage, and subsequently contributes to induction of apoptosis. Thus caffeine promotes the cytotoxic effect of ionising radiation and provides a platform for the development of new anti-cancer therapeutics known as radio-sensitisers.

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