Casein kinase 2 inhibition modulates the DNA damage response but fails to radiosensitize malignant glioma cells

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Abstract. Inhibitors of casein kinase 2 (CK2), a regulator of cell proliferation and mediator of the DNA damage response, are being evaluated in clinical trials for the treatment of cancers. Apigenin was capable of inhibiting the activation of CK2 following γ irradiation in LN18 and U87 malignant glioma cells. Apigenin and siRNA-mediated CK2 protein depletion further inhibited NF-κB activation and altered the Tyr68 phosphorylation of Chk2 kinase, a DNA damage response checkpoint kinase, following irradiation. However, CK2 inhibition did not decrease the ability of these glioma cells to repair double-strand DNA breaks, as assessed by COMET assays and γ-H2Ax staining. Likewise, apigenin and siRNA-induced depletion of CK2 failed to sensitize glioma cells to the cytotoxic effect of 2 to 10 G-rays of γ irradiation, as assessed by clonogenic assays. These results contrast with those found in other cancer types, and urge to prudence regarding the inclusion of malignant glioma patients in clinical trials that assess the radiosensitizing role of CK2 inhibitors in solid cancers.

Introduction

Malignant astrocytomas represent the most frequent type of malignant brain tumors and are characterized by a strong resistance to therapies and a dismal prognosis (1). Among the factors that determine this resistance to treatment, anti-apoptotic signaling, for instance through constitutive NF-κB pathway activation (2,3), and altered DNA-damage response (4), are believed to play major roles.

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Casein kinase 2 (CK2), an ubiquitous serine threonine kinase, consists of tetramers of 2 catalytic and 2 regulatory subunits. It has recently gained interest in the field of cancer research as both a regulator of proliferation and survival pathways and a modulator of the DNA-repair machinery (5,6). CK2 was thus shown to regulate NF-κB and STAT3 activation, P53 function (7), PTEN activity, Akt-dependant signaling, mTOR stability and SIRT-dependent protein acetylation (6,8-13). CK2 also regulates the function of several enzymes of the DNA-repair and DNA-damage sensing machinery, such as XRCC1 and 4, Rad9 and DNA-PK (14-16). As a corollary, pre-clinical studies have shown that CK2 inhibitors elicit anti-tumoral effects against leukemias, prostate carcinomas, breast cancers, and some PTEN or TP53 mutated malignant gliomas (17,18). Based on these reports, CK2 inhibitors entered the field of clinical trials (8,19-22). Among them, apigenin is a naturally occurring plant flavonoid and a specific inhibitor of the catalytic subunits of CK2 (8,23,24). It was shown to reduce the proliferation and to induce apoptosis in several carcinoma cells (25-27) as well as in some human glioma cell lines (28), and has recently been used in a phase II trial for the prevention of colorectal cancer recurrence (NCT00609310).

Given this growing interest of clinicians and the industry for CK2 inhibitors, and in view of the fundamental yet disappointing role of radiation therapy for the treatment of malignant gliomas (1), we investigated whether CK2 inhibition would alter the radiation-induced DNA repair response and whether these tumors could be radiosensitized.

Materials and methods

Cell cultures, reagents and siRNA. Cell lines U87 and LN18 were obtained from the American Type Culture Collection (ATCC) and grown in DMEM (Dulbecco's modified Eagle's medium, Gibco, Gent, Belgium) containing 10% of fetal bovine serum (FBS, Gibco) and penicillin. Cultures were maintained at 37˚C under a humidified atmosphere containing 5% carbon dioxide.

Apigenin was purchased from Sigma (Bornem, Belgium), dissolved in dimethylsulfoxide (DMSO) and used at final concentration of 40 μM (stock solution, 100 mM). Control cells were treated with a similar final concentration of DMSO as the

apigenin-treated cells. Irradiations of cell lines were conducted with a research irradiator (Gammacell 40 Exactor, Theratronics, Stockley Park, UK).

Subconfluent cultured cells were transfected with 50 nmol/l ON-TARGETplus non-targeting pool or SMARTpool human CSNK2A1 siRNA from Dharmacon (Fisher Scientific, Tournai, Belgium) using oligofectamine (Invitrogen, Gent, Belgium) according to the manufacturer's instructions. Cells were harvested and assayed 48 h after transfection. CK2 depletion was controlled using western blot analysis of the expression of CK2-α.

CK2 and IKK-β kinase assays. Cells were lysed using RIPA buffer extraction kit (Santa Cruz Biotechnology) and 300 μg of protein were taken for immunoprecipitation. After a pre-cleared step, supernatant were incubated with an anti-CK2 antibody (clone 1AD9, Millipore, Overijse, Belgium) under rotary agitation for 4 h at 4˚C. GammaBind G Sephorase beads (25 μl/samples, GE Healthcare, Diegem, Belgium) were then added to the sample and incubated on a rotating system overnight at 4˚C. After three washes, immunoprecipitated proteins were processed with the CK2 assay kit (Upstate, Millipore) or the IKK-β kinase assay kit (Cell Signaling, Bioke, Leiden, The Netherlands), according to the manufacturer's instructions.

NF-κB transcription assay. Cells were co-transfected by using TransIT-2020 transfection reagent (Mirus, Eke, Belgium) with: i) a luciferase-coupled reporter gene for NF-κB and ii) a *Renilla* luciferase reporter driven by a constitutive promoter. Radiation (10 Gy) and apigenin treatment (40 μM) effects on NF-κB transcriptional activity were assessed 24 h later. Briefly, cells were lysed and luciferase activities were measured according to the manufacturer's instructions for the Dual Luciferase Assay System (Promega, Leiden, The Netherlands) and using a Victor luminometer (PerkinElmer, Zaventem, Belgium). The relative NF-κB luciferase activity was normalized to the one of the *Renilla*.

Western blot analysis. 10% polyacrylamide precast gels (Mini Protean TGX, Bio-Rad, Nazareth Eke, Belgium) were run for 30 min at 200 volts with nuclear extract (20 μg/well) obtained from irradiated and previously apigenin or DMSO treated cells. Protein extracts were obtained using conventional RIPA buffer and phosphatase inhibitors. After transfer to a PVDF membrane (Roche, Vilvoorde, Belgium) for 2 h at 300 mA and blocking with Tris buffered saline containing 0.2% Tween plus 5% dry milk powder, membranes were incubated overnight at 4˚C in the presence of primary antibody directed against phospho(Thr68)-Chk2 (Cell Signaling, Bioké, Leiden, The Netherlands). A horseradish peroxidase-coupled secondary antibody was then incubated, and peroxidase activity was evidenced with the Super Signal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Aalst, Belgium) and the ImageQuant LAS 4000 Mini Biomolecular Imager (GE Healthcare).

Cell survival assays. Cell survival in response to apigenin treatment and radiation was assayed using clonogenic assays and MTS tests (One Solution Cell Proliferation Assay, Promega). Clonogenic assays were performed on cells plated at low-density as described previously (29) and as recommended by the manufacturer's protocol for MTS assays.

DNA repair assays. Single-cell gel electrophoresis under alkaline conditions and flow cytometry measurement of phosphorylated γ-histone 2Ax (γ-H2Ax) foci were used to identify ds-DNA breaks and associated repair mechanisms, respectively.

ds-DNA breaks following apigenin (or DMSO) treatment and radiation were detected by using the CometAssay HT kit (Trevigen, Sanbio, Uden, The Netherlands). Briefly, single cells embedded in agarose were lysed to remove proteins and where then submitted to electrophorese. Staining was performed with SYBR green I (Trevigen) for 15 min. The slides were examined under a fluorescent microscope (Zeiss Axiovert 10 microscope, Carl Zeiss) and DNA tail lengths were quantified in a blinded manner by counting a minimum of 50 cells per condition in independent experiments.

Treated cells were harvested at different times and were prepared for flow cytometer analysis. Approximately $2.5x10^6$ cells/ml were resuspended in 250 μl of PBS and fixed by adding the same amount of 4% paraformaldehyde (PFA, Merck, Overijse, Belgium). After permeabilization and blocking with PBS containing 0.5% Triton X-100 (Acros Organics, Geel, Belgium) and 5% donkey serum (Jackson Immunoresearch Laboratories, Newmarket, UK) for 20 min, an anti-phosphorylated Ser139 γ-H2Ax mouse monoclonal antibody (1:500, Millipore) was incubated for 90 min at room temperature. Three PBS washes later, we incubated cells with an FITC-conjugated secondary antibody (1:500, Jackson Immunoresearch Laboratories). Indirect immunofluorescence staining was immediately analyzed after three more PBS washes (FACS Calibur, BD Biosciences, Erembodegem, Belgium).

Statistical analysis. Statistical analyses were performed using the Prism 5.0c for Mac software (Graphpad Inc., La Jolla, CA). One-way ANOVA and Mann-Whitney U tests were performed when appropriate and as described in the results section.

Results

Irradiation-induced CK2 kinase activity in malignant glioma cells. Exposure of LN18 and U87 cells to ionizing radiations (γ rays, 4 Gy) increased the catalytic activity of CK2 within 30 min, by respectively 25±5% and 45±2.5%. Both the basal and radiation-induced CK2 activities were significantly abolished by pretreatment of the cell cultures with 40 μM Apigenin for 1 h (mean ± SD, n=3, $P<0.05$ for both, ANOVA with Tukey's post tests; Fig. 1).

Irradiation-induced NF-κB activation in malignant glioma cells. Ionizing radiation activates NF-κB in tumors and glioblastomas via an ATM-NEMO-IKK-kinase dependent pathway (30). UV-induced DNA damage, however, also activates CK2 (31), leading to an IKK-kinase-independent C-terminal phosphorylation and degradation of I-κBα, and NF-κB activation (32). In LN18 and in U87 cells, ionizing irradiation (10 Gy) increased within 1 h the activity of an NF-κB-driven luciferase reporter gene by 31±6.6% and 66±34%, respectively, (mean ± SD, n=3, $P<0.05$, one-way ANOVA with Tukey's post tests). The baseline activity of the reporter gene was inhibited following apigenin treatment and remained significantly reduced in these cells despite irradiation ($P<0.05$, 40 μM, Fig. 2).

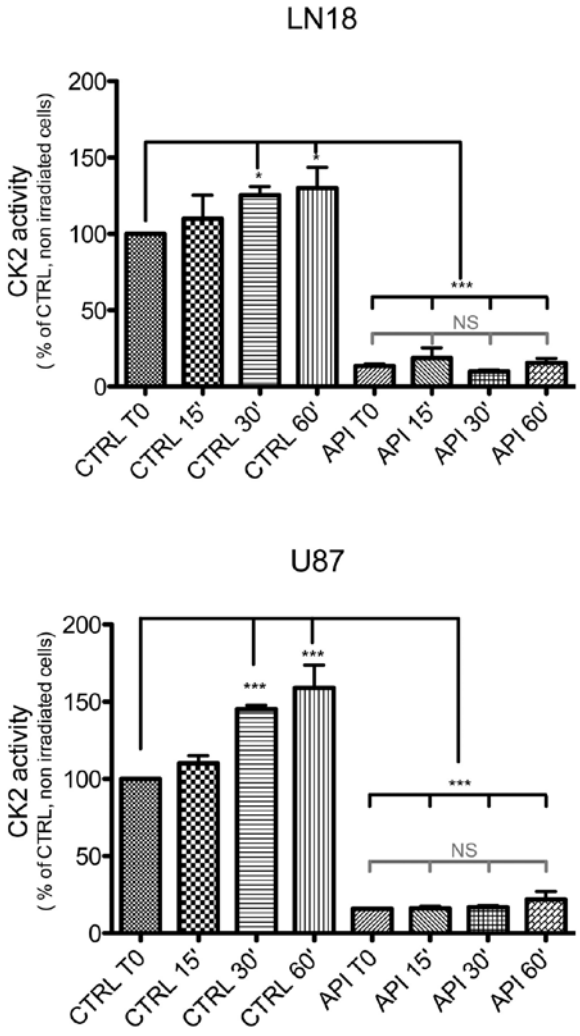


Figure 1. CK2 kinase assays following irradiation. LN18 and U87 cells were irradiated (4 Gy), homogenized and assessed for CK2 kinase activity following immunoprecipitation of the CK2 complex, as explained in the Materials and methods section. CTRL, cells are treated with DMSO only; API, cells are treated with 40 μM apigenin in DMSO, for 1 h prior to the irradiation. Experiments are represented as percents of control, non-irradiated cells (T0), and as the mean ± SD of 3 independent experiments. (*P<0.05; ***P<0.001, ANOVA with Tukey's post tests).

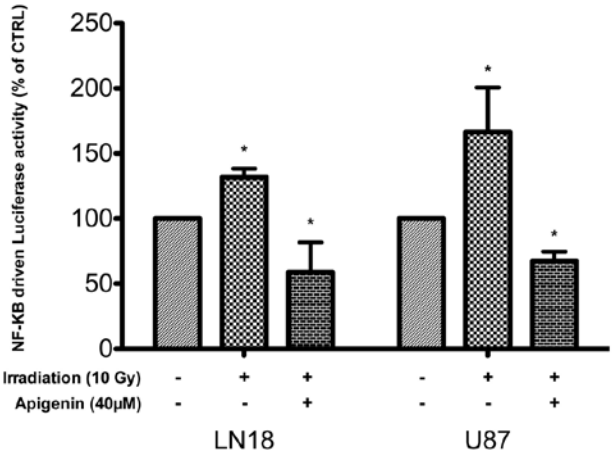


Figure 2. NF-κB reporter gene assays. NF-κB-driven luciferase activity was measured 1 h after irradiation (10 Gy) prior and after irradiation of LN18 and U87 cells in control (DMSO) conditions and following apigenin treatment. Activities are represented in percents of control, non-irradiated cells, and are shown as the mean ± SD of 3 independent experiments (*P<0.05, ANOVA with Tukey's post tests).

CK2 inhibition and DNA-repair in malignant glioma cells. CK2 has recently emerged as a regulator of the DNA damage response machinery (33). We thus performed COMET assays to measure the influence of CK2 inhibition on ds-DNA break formation in U87 and LN18 cells following γ irradiation (10 Gy). As shown in Fig. 3A, si-mediated CK2 depletion slightly decreased the peak amplitude of COMET tails in LN18 cells 3 h following a 10 Gy irradiation (P<0.05, Mann-Witney U test). It however had the opposite effect in U87 cells (P<0.05, Mann-Witney U test). The mean tail amplitude returned to baseline in mock-treated and siCK2-treated LN18 cells after 24 h. Tail size also returned to baseline in siCK2-treated U87 cells, in sharp contrast to mock-transfected cells where tails still remained significantly longer than at baseline at this time point (P<0.0001, Mann-Witney U test).

We also assessed the kinetics of γ-H2Ax foci formation in LN18 and U87 cells treated with apigenin (40 μM) using FACS cytometry. In both cell types, radiation treatment (10 Gy) increased the amount of γ-H2Ax immunoreactivity with respect to baseline conditions, with a peak within 1 to 3 h. γ-H2Ax signal returned towards baseline in control and apigenin-treated in both cell types within 24 h. Apigenin treatment did not alter these post-irradiation kinetics of γ-H2Ax immunoreactivity (data not shown).

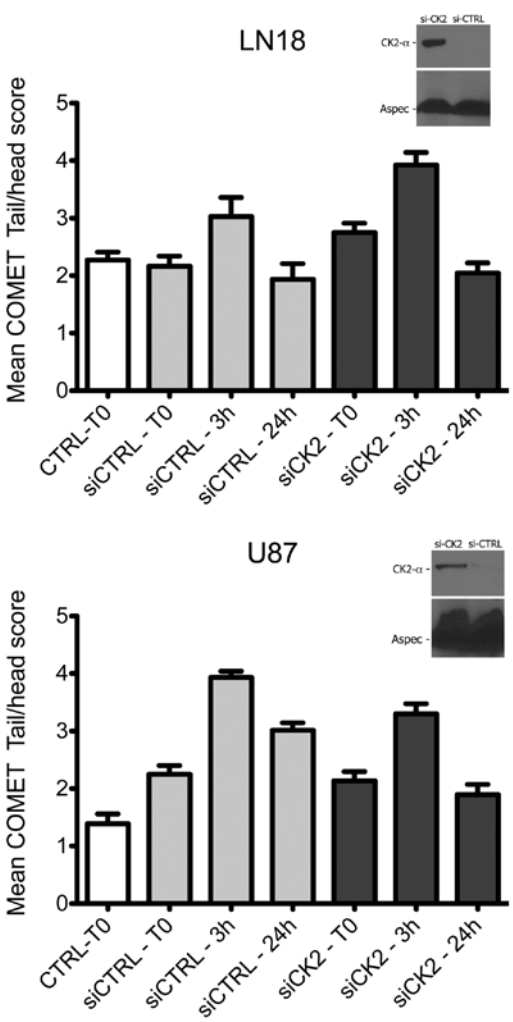
CK2 is also known to inhibit the DNA-repair kinase DNA-PK (15), and the Chk2 checkpoint kinase is phosphorylated on tyrosine 68 by DNA-PK following irradiation (34). Tyr68 phosphorylation of Chk2 was induced in LN18 and U87 within 15 min after irradiation (4 Gy). This event was potentiated and more durable in both cell types by a pre-treatment with 40 μM apigenin (Fig. 3B).

CK2 inhibition and cell survival following γ irradiation. Both U87 and LN18 cells displayed a moderate but significant reduction in viability following 4 G-rays of γ irradiation (25.5±13.2% and 27±19.5%, respectively, P<0.05, one-way ANOVA) as assessed using an MTS test (Fig. 4A). This viability was not further reduced following co-treatment with 40 μM apigenin. At this concentration, apigenin treatment also failed to radiosensitize U87 and LN18 cells in clonogenic assays (Fig. 4B, upper pannel).

As CK2-independent effects of apigenin have been reported (35), we also assessed the effect of siRNA-mediated CK2 kinase depletion on the radiosensitization of malignant glioma cells. The clonogenic survival of U87 and LN18 cells treated with CK2-targetting siRNA prior to irradiation did not differ from scramble siRNA treated controls (Fig. 4B, lower pannel).

Discussion

CK2 has recently appeared as a regulator of ds-DNA break (DSB)-triggered signaling cascades in normal, carcinoma and even in some malignant glioma cells (36).



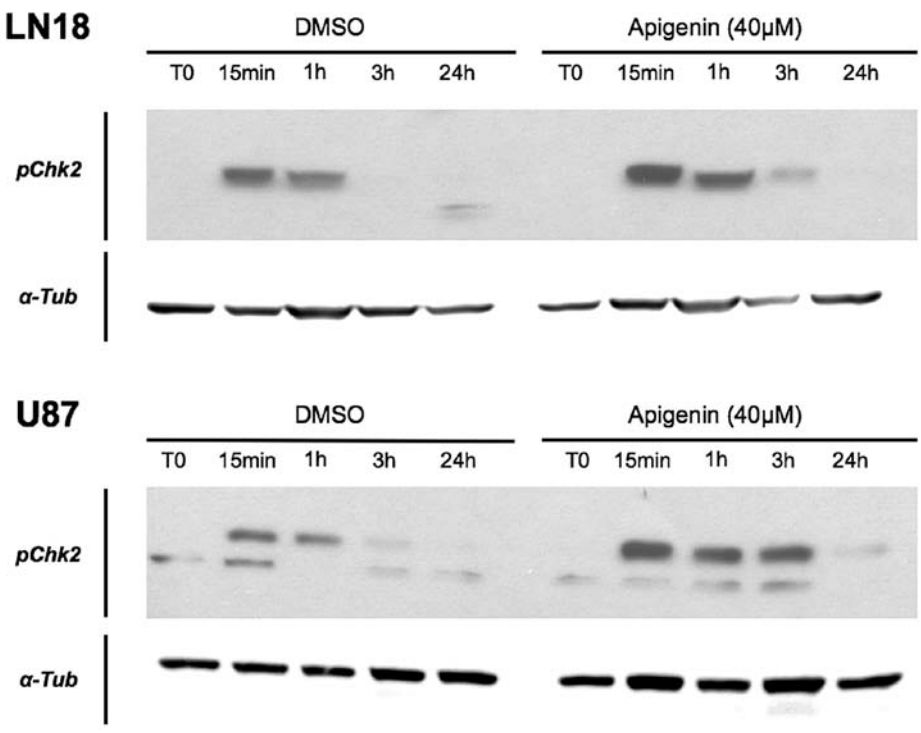


Figure 3. CK2 inhibition and DSB-repair. (A) COMET assays were performed on glioma cells transfected with siRNA to CK2 or control scramble siRNAs, and then irradiated (10 Gy). ds-DNA break formation was determined 3 h and 24 h after the irradiation. COMET tail/head ratios are shown as the means ± SD of a minimum of 50 cells (*P<0.05; ****P<0.0001, Mann-Witney U test). (Inserts): Western blot analysis of the inhibition of CK2-α expression 48 h after the siRNA transfection. (B) Western blot analysis of Tyr68 phosphorylation of CK2 in U87 and LN18 cells following irradiation (4 Gy) and apigenin treatment (40 μM).

Accordingly, CK2-α, the active kinase subunit of CK2, was activated within minutes of radiation treatment in our malignant glioma cells. siRNA-mediated CK2 depletion significantly increased the maximal peak of DSB in LN18 cells, but not in U87 cells. However, CK2 knock-down did not inhibit the repair of ds-DNA breaks in our cell lines, but even slightly improved it, as evidenced by the normalization of COMET assays within 24 h after irradiation in both cell lines and the faster return of γ-H2Ax immunoreactivity towards the baseline in U87 cells following apigenin treatment.

In standard conditions, homologous recombination (HR) only plays an accessory role in ds-DNA repair following ionizing radiation in gliomas, and these tumors rather proceed via non-homologous end joining (NHEJ) (37). During NHEJ, CK2 phosphorylates XRCC4 and helps recruit repair enzymes like PNKP and APLF to this scaffold protein (13,15,36,38,39). According to this, and in contrast to our COMET and γ-H2Ax findings, CK2 inhibition should thus inhibit DNA repair. In better agreement with our results however, CK2 inhibition did not impair ds-DNA rejoining in fibroblasts or colon carcinoma cells (40). As a tentative explanation, CK2 also inhibits the DNA-dependent protein kinase subunit DNA-PKcs in glioblastomas (15). DNA-PK is itself a key inhibitor of HR (41), and gliomas might thus escape CK2 inhibition-induced NHEJ inhibition via an increase in homologous recombination. In agreement with this hypothesis, apigenin treatment of glioma cells increased the radiation-induced phosphorylation of the DNA-PK target Chk2 (34,42) in our experiments.

In colon carcinoma cells and fibroblasts, although CK2 inhibition does not alter the rejoining of DSB, it does slow down

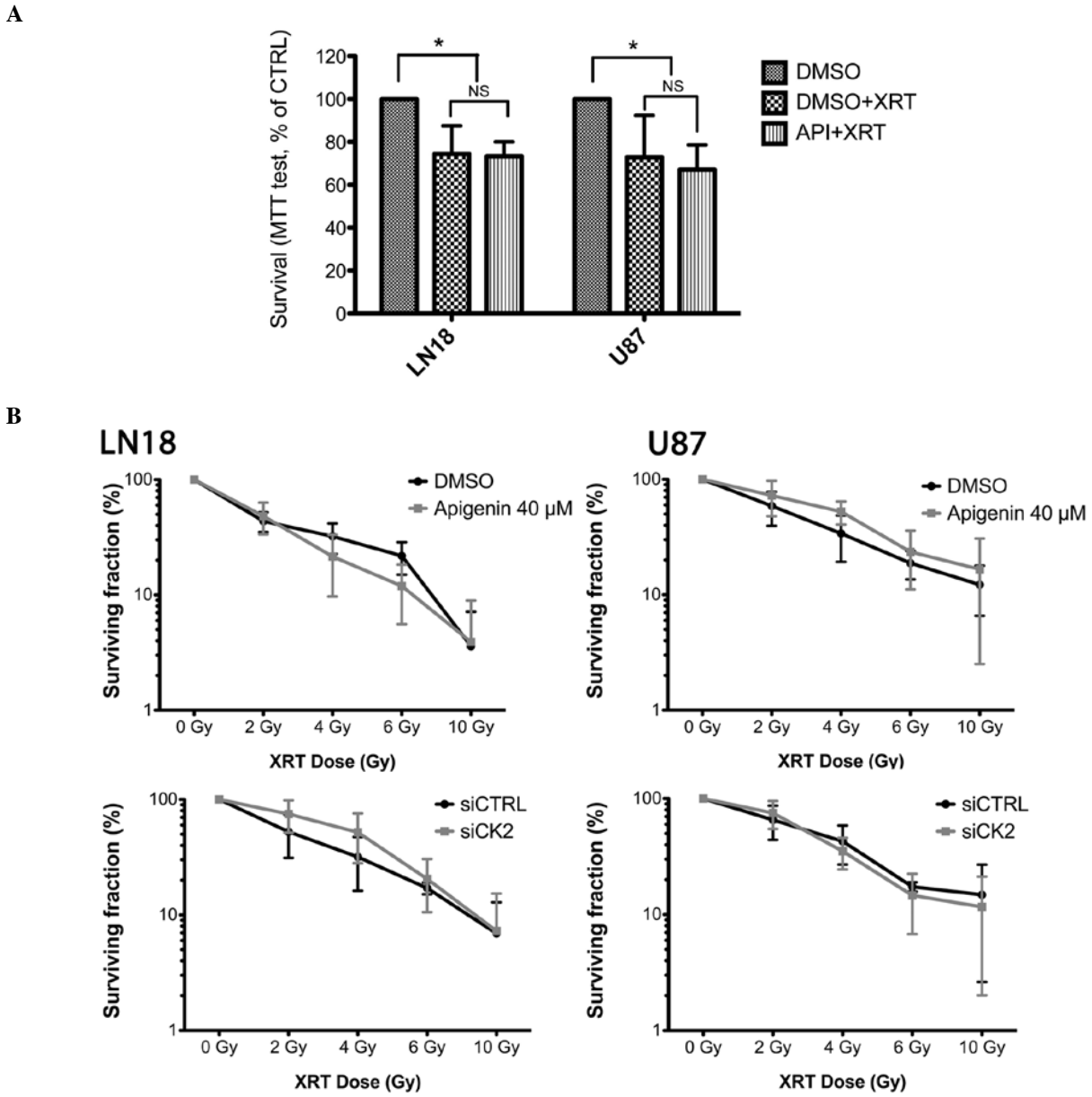


Figure 4. CK2 inhibition and survival of glioma cells following irradiation. (A) MTS proliferation assays were performed 5 days after a 4 Gy irradiation (XRT) following a 1 h pre-treatment with 40 μM apigenin. Experiments are represented in percents of control, non-irradiated cells, and as the mean ± SD of 3 independent experiments (*P<0.05, ANOVA with Newman-Keuls post tests). (B) Clonogenic assays of LN18 And U87 cell survival 9 days after irradiation (2, 4, 6 and 10 Gy) in the presence or not of apigenin (upper panel) or after CK2-α depletion (lower pannel). Results are expressed in percents of controls, non-irradiated cells and as the mean ± SD of 4 independent experiments.

the dephosphorylation of γ-H2Ax and its dissociation from the DNA after repair (40). Such a lengthened γ-H2Ax decay is believed to amplify checkpoint signaling in the presence of minimal residual DNA damage and lead to cell death (43). We did not observe this phenomenon in our experiments.

In our experiments and in agreement with previous reports (44,45), CK2 inhibition also reduced the constitutive level of NF-κB reporter activity in both cell lines. The post-radiation transcriptional activity of this factor also remained significantly lower in apigenin-treated irradiated cells than in the control, non-irradiated cells. Apigenin-treated cells, however, still responded to irradiation with a minimal induction of NF-κB (data not shown), and our results thus do not contradict the paradigm that CK2 triggers NF-κB activation in response to UV-induced DNA damage but not following exposure to ionizing radiation (46-49).

Pharmacological NF-κB inhibitors are nonetheless known to modulate the fate of tumor cells following irradiation. They were reported to radiosensitize glioblastomas (50,51), but NF-κB was also, on the contrary, recently reported to mediate apoptosis following the irradiation of primary cultures and progenitor cells of gliomas lines (52). In line with these contrasting reports and its favorable effect on DSB-repair in our experiments, CK2 inhibition did not radiosensitize our glioma cells. This neutrality seems to be independent of TP53 mutational status, as we confirmed by exon sequencing that LN18 and U87 cells express, respectively mutant and wild-type variants of this CK2 target (data not shown) (53-55). Aspecific effects of apigenin were

also ruled out by repeating clonogenic assays following siRNA mediated depletion of CK2-α.

Although we cannot rule out that CK2 inhibition could radiosensitize glioblastoma cells with defective DNA-PK, the lack of radiosensitization of gliomas that we have observed contrasts with that of non-small cell lung carcinomas, fibroblasts and colon carcinomas cells following CK2 inhibition (6,40). Since DNA-PK mutations occur in only 3% of glioblastomas (TCGA data portal, accessed January 16th, 2011; the TCGA research network) (56) we believe that glioma patients should not be included in clinical trials that assess the radiosensitizing role of CK2 inhibitors. Further studies of DNA repair mechanisms in primary brain tumors and a preclinical evaluation of therapies combining CK2 inhibitor with other DNA-damaging agents with DNA-PK inhibitors are required to improve the therapeutic options for these tumors.

In spite of its modulation of DNA-damage signaling cascades, CK2 inhibition fails to inhibit DNA repair following ionizing radiation and to radiosensitize glioma cells, independently of their TP53 status. This contrasts with other tumor types, urging caution regarding the inclusion of malignant glioma patients in clinical studies that will assess the radiosensitizing role of CK2 inhibitors in solid cancers.

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