Cyclin-dependent Kinase (CDK) Inhibitor Olomoucine Enhances γ-Irradiation-induced Apoptosis and Cell Cycle Arrest in Raji Cells

ANASTASIOS BOUTIS[1,2,3], KONSTANTINOS PAPAZISIS[1,2], KYRIAKI PISTEVOU-GOMPAKI[3], ALEXANDROS LAMBROPOULOS[4], IOANNIS SOFRONIADIS[3], ATHANASIOS PAPAGEORGIOU[2], EVANGELIA DESTOUNI[5] and ALEXANDROS KORTSARIS[6]

[1]Third Department of Clinical Oncology, [2]Symeonidion Research Centre and
[5]Department of Radiotherapy, Theagenion Cancer Hospital, Thessaloniki 54007;
Departments of [3]Radiotherapy and [4]Biology Medical Faculty, Aristotle University of Thessaloniki, Thessaloniki;
[6]Department of Biochemistry, Medical Faculty, Democritus University of Thrace, Alexandroupolis, Greece

Abstract. *Gamma-irradiation leads to apoptosis and cell cycle arrest in eukaryotic cells. Olomoucine is a novel purine analog acting as a cyclin-dependent kinase inhibitor. The effects of olomoucine in gamma-irradiation mediated cell growth inhibition and apoptosis were studied in the Raji cell line (Burkitt's lymphoma). Gamma-irradiation caused a G_2 arrest, increasing the G_2/M fragment of the cells. Apoptosis by gamma-irradiation was apparent both by DNA-electrophoresis and PARP-1 cleavage. The combination of olomoucine with irradiation caused an increased G_2 arrest and decreased cell survival and DNA synthesis in the non-apoptotic fraction of the remaining cells. Irradiation, as well as olomoucine and the combination of both, induced apoptosis. It seems that olomoucine delays the apoptotic process and inhibits DNA fragmentation, but it decreased survival, cell cycle progression and proliferation of irradiated cells.*

Gamma-irradiation leads to apoptosis and cell cycle arrest in eukaryotic cells (1). The signal transduction of apoptosis and/or cell cycle arrest is believed to involve activation of a DNA-damage checkpoint that leads to chk1/chk2 activation, p53-dependent gene expression, CDK1/Cyclin-B inactivation (G_2-phase arrest), caspase activation and PARP (poly-ADP-ribosyl polymerase) cleavage (1). Gamma-irradiation-induced G_2 arrest is believed to allow cells to repair DNA before entering mitosis, thus, protecting cell viability. Abrogation of the G_2 checkpoint leads to enhanced apoptosis after γ-irradiation in cells that lack normal p53 function (2) and in K562 erythroleukemia cells (3).

Olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) is a purine analog that exhibited a strong inhibitory effect against CDK-1/Cyclin-B, CDK-2/Cyclin-E, CDK-2/Cyclin-A, CDK-5/p35 and ERK-1 (4). Olomoucine was shown to inhibit cell death induced by paclitaxel in MCF-7 cells (5) and by cytosine-arabinoside in HeLa cells (6). It also inhibited programmed cell death in PC12 cells and sympathetic neurons deprived of trophic support (7) or treated with DNA-damaging agents (8). Olomoucine prevented apoptosis after withdrawal of neurotrophic support from neuronal cells, an action that cannot be attributed to its ERK-1 inhibitory activity (9).

The Raji cell line was derived from a patient with Burkitt's lymphoma (10). It is the first continuous human cell line of hematopoietic origin. As Raji cells are of a Burkitt's leukemia origin, they have been transformed with the Epstein-Barr virus (EBV). The effects of olomoucine in γ-irradiation-induced cell growth inhibition and apoptosis were studied in the Raji cell line *in vitro*.

Materials and Methods

Cell lines and culture conditions. The Raji cell line (Burkitt's lymphoma) was obtained from the Imperial Cancer Research Fund (ICRF, London, UK). The cells were grown as suspension cultures in T-75 flasks (Costar-Corning, Cambridge, UK) at 37˚C in an atmosphere containing 5% CO_2 in air and 100% relative humidity and were passaged twice a week and maintained at a low passage number (5-20). The culture medium used was RPMI-1640 (Gibco, Glasgow, UK), supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Sigma

Correspondence to: Anastasios Boutis, MD, Theagenion Cancer Hospital, 3rd Department of Clinical Oncology, Al. Symeonidi Str. 2, Thessaloniki 54007, Greece. Tel: +30 2310 898720, Fax: +30 2310 898753, e-mail: tboutis@otenet.gr

Key Words: Cyclin-dependent kinase inhibitor, olomoucine, γ-irradiation, apoptosis, cell cycle arrest, Raji.

Chemicals Co., St. Louis, USA), 100 μg/ml streptomycin and 100 IU/ml penicillin.

Chemicals. Olomoucine was purchased from Sigma Chemicals and was dissolved in DMSO (Sigma) to a concentration of 0.1 M. The final concentration of DMSO in culture was less than 0.1%, a concentration that exhibits no effect on cell growth and proliferation, as experimentally confirmed.

Irradiation. Cells were irradiated under a ^{60}Co source (Theratron 780E, CIS-Int., Canada) at a distance of 80 cm from the source and a ratio of 2.5 Gy/min. The total dose varied between 2.5 and 10 Gy in preliminary experiments and a dose of 5 Gy was thereafter adopted as an effective dose that inhibited 50% of cellular survival and could induce apoptotic cell death.

Treatment planning. For all experimental procedures, 10x10^6 cells were seeded in 75 cm^2 flasks containing 30 ml of growth medium. Treatment was planned as follows: control flasks were kept out of the incubation chamber for the same time needed for irradiated cells to complete irradiation (a few minutes only) and cultured thereafter for a period between 24 to 72 h. Some flasks were exposed to 50 μM of olomoucine for 24 to 72 h (these flasks were also kept out of the incubation chamber for the same time needed for irradiated cells to complete irradiation). Gamma-irradiated flasks were cultured for 24 to 72 h after irradiation. Finally, combination flasks were exposed to 50 μM olomoucine irradiated 30 min later and subsequently cultured for 24 to 72 h in the presence of the same concentration of olomoucine.

For cell cycle analysis, 3x10^6 cells from each experiment were fixed (11) and kept in 4˚C for a maximum period of 4 days (short-term storage does not alter results, as was experimentally confirmed). The content of cellular DNA was assessed with propidium iodide (12, 13). To avoid an increased signal from staining, artifact cells were digested with DNase-free RNase A (14). Cellular DNA content was measured using an Epics II flow cytometer (Coulter, El) using the Multicycle Cell Cycle Analysis Software (Phoenix Flow Systems Inc.).

DNA-electrophoresis. Apoptosis was evaluated by electrophoresis in agarose gel, after exposure of cells (grown in 75-cm^2 flasks) to olomoucine, γ-irradiation or both. DNA was extracted from the cells 24 h after irradiation, using a commercially available kit (Gentra Systems, MN, USA). Equal amounts of DNA were loaded into the wells of an agarose gel and electrophoresed for 2 h at 100 V at room temperature. Ethidium bromide staining and a 312 nm UV-transilluminator (Spectroline) were used for visualization.

Western blot. Approximately 2x10^6 cells from each experiment were lysed in "RIPA Buffer" (0.5% Sodium Deoxycholate, 1% Nonidet P40 and 0.1% SDS in PBS) containing a cocktail of protease and phosphatase inhibitors (PMSF, Aprotinin and Sodium orthovanadate). After centrifugation (12,000 xg for 15 min at 4˚C), the supernatant was assayed for protein using the Bio-Rad protein Assay (Bio-Rad, CA, USA) that is based on the method of Bradford (15). Subsequently, 20 μg of protein were boiled with 20% sample buffer (250 mM Tris [pH 6.8], 10% SDS (sodium dodecyl sulphate), 50% glycerol, 6.25% v/v 2-mercaptoethanol and 0.06% w/v bromophenol blue) for 5 min, loaded in a 10-12% SDS-polyacrylamide gel, electrophoresed and transferred to Immobilon-P polyvinylidene difuride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked with 0.1% Tween-20 and 5% Bovine Serum Albumin (BSA, Sigma) in PBS for 2 h and then incubated for 45 min with primary antibody (rabbit polyclonal anti-PARP-1, Calbiochem) and 45 min with secondary (HRP-labelled) antibody (Amersham). Finally, blots were developed using the chemiluminescence method (WestPico, Pierce).

Table I. *Cell cycle distribution of Raji cells 24 h after initiation of the experiment.*

	G_1 (%)	S (%)	G_2/M (%)
Control	39.6	38.6	21.8
Olomoucine 25 μM	42.7	32.8	24.5
Olomoucine 50 μM	43.9	34.5	21.6
γ-Irradiation 5 Gy	32.0	37.2	30.8
γ-Irradiation 5 Gy & Olomoucine 25 μM	33.5	28.0	38.5
γ-Irradiation 5 Gy & Olomoucine 50 μM	37.0	23.4	39.6

Apoptotic cell separation. Apoptotic cells were excluded by negative selection with annexin-V labelled magnetic beads using MACS Apoptotic Cell Isolation kit (Miltenyi Biotec, UK). Annexin-V magnetic micro beads bind specifically to cells that have redistributed phosphatidylserine in the cytoplasmic membrane, *e.g.,* apoptotic cells (16). Cells were subsequently passed through a separation column placed in a strong magnetic field. The magnetically labelled cells were retained in the column, while the unlabelled live cells passed through and remained as the "negative" fraction. After removal of the column from the magnetic field, the magnetically retained apoptotic or necrotic cells were eluted, comprising the "positive" fraction.

DNA synthesis estimation. DNA-synthesis was estimated only in live (not apoptotic or necrotic) cells by the BrdU assay (17) using a standard colorimetric ELISA, as previously described (3), after exposure to 10 μM BrdU for the last 60 min of the experiment.

Results

Cell cycle distribution. Cells were cultured in the presence of two different concentrations of olomoucine (25 μM and 50 μM) for 30 min and were then irradiated at a dose of 5 Gy and subsequently incubated for 24 h. Exposure to the drug or to irradiation or both resulted in alterations in cell cycle distribution, which were evaluated with flow cytometry (Figure 1, Table I).

Gamma-irradiation induced a G_2 cell cycle arrest increasing the G_2/M fraction. Olomoucine did not have significant effect upon cell cycle distribution in the two concentrations under evaluation. However, the combination of olomoucine with irradiation caused an increased G_2 arrest. The G_2 arrest was combined with a decreased S-phase and an increase of apoptotic cells (pre-G_1 signal in Figure 1).

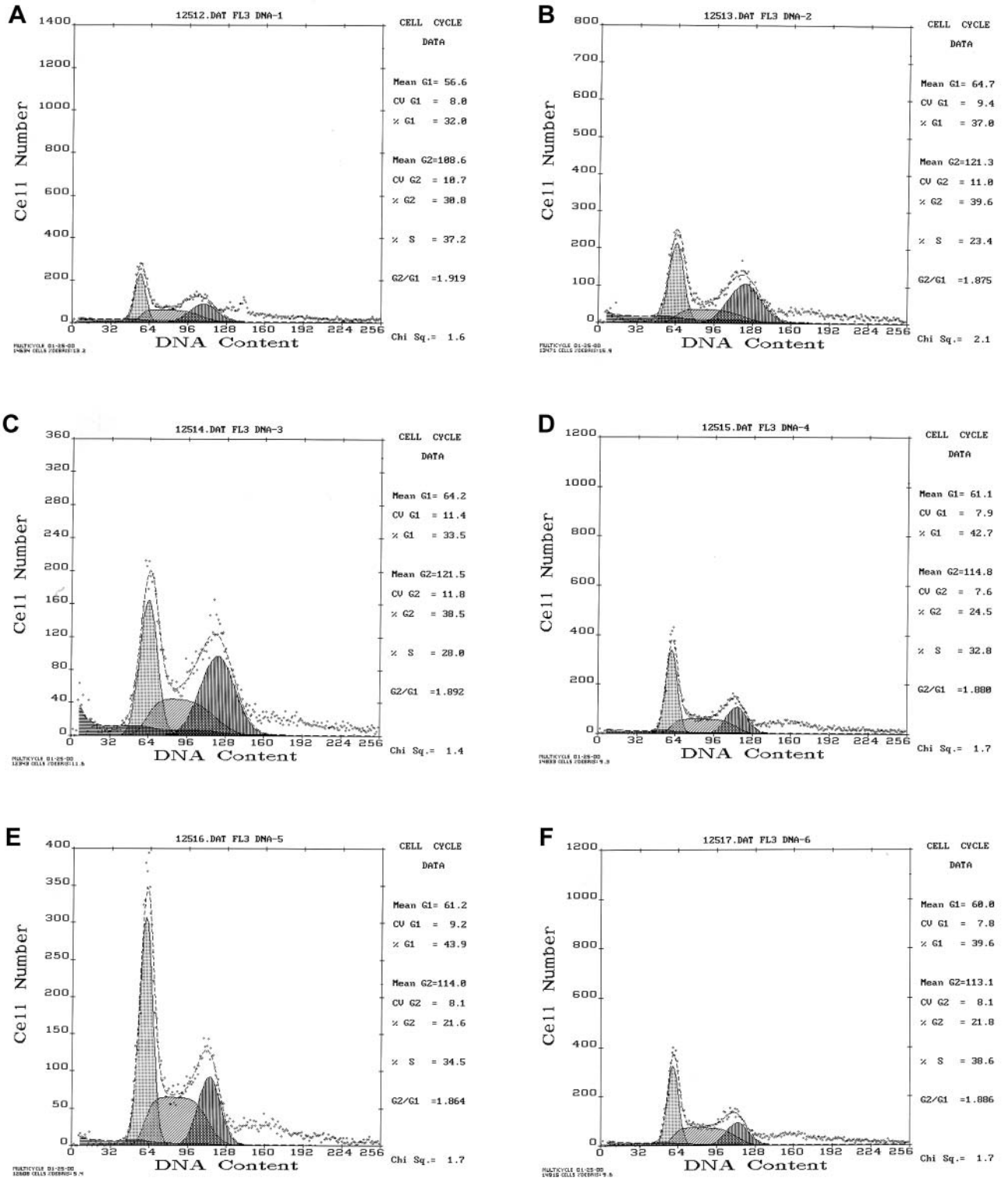

Figure 1. *Flow cytometry analysis of cell cycle distribution of Raji cells: (A) control Raji cells, (B) Raji cells treated with olomoucine 25 μM for 24 h, (C) Raji cells treated with olomoucine 50 μM for 24 h, (D) Raji cells 24 h after γ-irradiation with 5 Gy, (E) Raji cells 24 h after γ-irradiation with 5 Gy and olomoucine 25 μM, (F) Raji cells 24 h after γ-irradiation with 5 Gy and olomoucine 50 μM. Cell number and DNA content are presented in the y-axis and x-axis, respectively.*

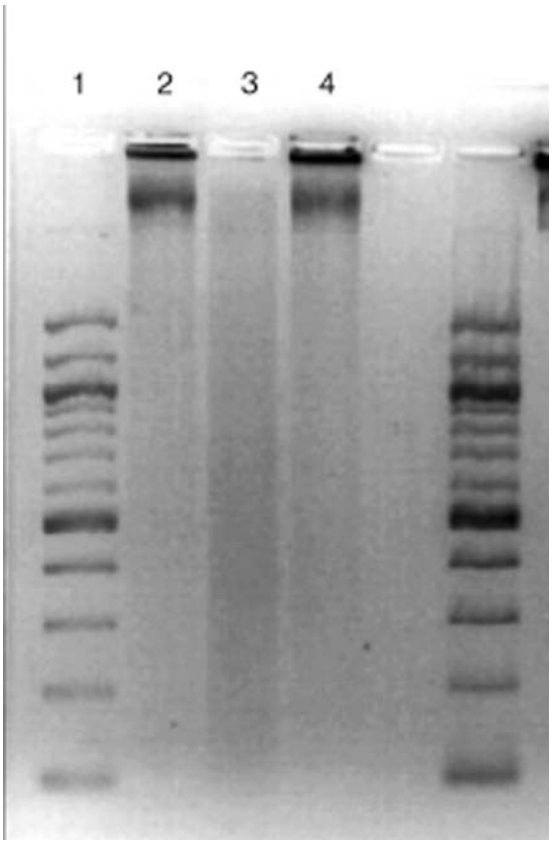

Figure 2. *DNA electrophoresis of Raji cells 24 h after treatment: (1) DNA ladder, (2) control, (3) γ-irradiation 5 Gy, (4) γ-irradiation and olomoucine 50 μM.*

DNA synthesis. DNA synthesis was evaluated 48 h after the exposure of the cells to olomoucine, irradiation or both, with BrdU incorporation assay. Each experiment was performed three times with 10^5 cells and the mean value was calculated. In order to avoid artefacts, apoptotic cells were excluded by negative selection with annexin-V labelled magnetic beads. Gamma-irradiation decreased DNA synthesis, as expected. The addition of olomoucine decreased cell survival and DNA synthesis in the live (annexin-V-negative selected) fraction of the remaining cells.

Apoptosis. After the exposure to olomoucine, irradiation, or both, the cells were incubated for 24 h. Subsequently, DNA from approximately $2x10^6$ cells for each experiment was electrophoresed in agarose gel, in order to evaluate DNA integrity. Although olomoucine was expected to induce apoptotic cell death in irradiated cells, DNA-electrophoresis did not confirm this hypothesis. Gamma-irradiation caused significant DNA fragmentation, while olomoucine completely reversed the phenomenon (Figure 2). This finding may be explained in two ways:

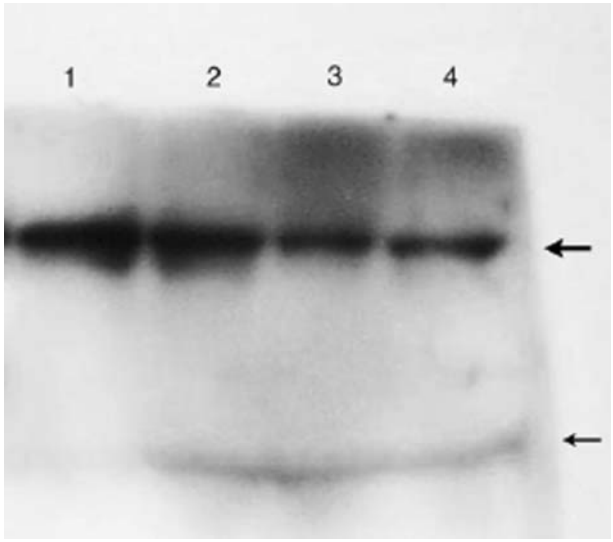

Figure 3. *PARP cleavage in Raji cells (Western blot): (1) control, (2) olomoucine 50 μM, (3) γ-irradiation 5 Gy, (4) γ-irradiation and olomoucine 50 μM. The bold arrow indicates the intact protein (113 kDa) and the regular arrow indicates the degradation product of PARP (24 kDa).*

I. Olomoucine inhibits cell cycle progression, thereby delaying apoptosis in M-phase. However, this delay will result in apoptotic cell death. DNA electrophoresis performed after 24 h cannot prove this phenomenon and prolongation of the experiment is impossible, because cells will undergo secondary necrosis.

II. Olomoucine alters the mechanism that results in apoptotic cell death.

The subsequent study of PARP cleavage with Western blot yielded the expected results. Irradiation as well as olomoucine and the combination of both induced apoptosis (Figure 3). It seems that olomoucine does not inhibit the apoptotic process, but delays the process and inhibits DNA fragmentation.

Discussion

DNA damage induced by factors such as γ-irradiation or chemotherapeutic drugs results in activation of p53 through an incompletely understood mechanism that involves possible activation of ATM and chk2 kinase. Both p53 and ATM are frequently mutated in human cancer; p53 is the most frequently mutated tumor suppressor gene in human cancer (18), indicating the importance of this checkpoint in cancer prevention. Chk1/chk2 kinases are extremely important components of the DNA-damage checkpoint and their inactivation (pharmaceutical or by inhibitory peptides) leads to abrogation of the G_2/M checkpoint, entrance to abnormal mitosis and apoptotic cell death in mitosis. Pharmaceutical

abrogation of the G_2/M is thus a very attractive target for anti-cancer drug development (19).

Exposure of cells to ionizing radiation induces DNA damage, which leads to cell cycle arrest or apoptosis in eukaryotic cells. Cell cycle arrest enables cell survival, allowing repair mechanisms to efficiently remove the damage and restore DNA integrity (3). Thus, agents that inhibit the DNA damage checkpoint are expected to favor apoptosis in irradiated cells.

Cyclin-dependent kinases (CDKs) are believed to be involved in the signal transduction of apoptosis after DNA damage. CDK inhibition by olomoucine (a purine analog that inhibit also ERK1) inhibited apoptotic cell death in some experimental models (6). However, olomoucine is also known to induce apoptosis in many cell lines and to block cell cycle progression in both G_1- and G_2-phases.

In our experiments, γ-irradiation produced a dose-dependent induction of apoptotic cell death (as confirmed by PARP cleavage and DNA-fragmentation) and G_2-phase cell cycle arrest in Raji cells. Olomoucine had little effect on cell cycle distribution and DNA synthesis when used alone. On the other hand, olomoucine induced apoptotic cell death (as it is shown in Figure 3, lane 2).

We previously showed that addition of olomoucine decreases cell survival after γ-irradiation in Raji cells (20). Addition of olomoucine to γ-irradiation inhibited apoptotic DNA fragmentation. However, PARP-1 cleavage was not altered significantly, indicating a negative effect of CDK-inhibitors in DNA fragmentation but not in the overall apoptotic process. DNA fragmentation is a downstream process after PARP-1 cleavage (21); olomoucine may interfere with this process without, however, altering the apoptosis *per se*.

The effect of olomoucine in cell cycle distribution was more straightforward. Although it had little effect on cell cycle phases when used alone, olomoucine increased the percentage of G_2-phase arrested cells after γ-irradiation and decreased overall survival. This can theoretically be explained by the effect of CDK inhibitors on normal cell cycle progression and DNA integrity. CDK-inhibitors abrogate the function of origins of replication (22), thus, further impairing the process of DNA synthesis and repair (23).

In conclusion, cyclin-dependent kinase inhibitor olomoucine altered DNA-fragmentation after γ-irradiation, but increased apoptotic cell death, decreased survival and proliferation and inhibited cell cycle progression.

References

1 Iliakis G: Cell cycle regulation in irradiated and nonirradiated cells. Semin Oncol *24*: 602-615, 1997.

2 Wang Q, Fan S, Eastman A, Worland PJ, Sausville EA and O'Connor PM: UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. J Natl Cancer Inst *88*: 956-965, 1996.

3 Papazisis KT, Zambouli D, Kimoundri OT, Papadakis ES, Vala V, Geromichalos GD, Voyatzi S, Markala D, Destouni E, Boutis L and Kortsaris AH: Protein tyrosine kinase inhibitor, genistein, enhances apoptosis and cell cycle arrest in K562 cells treated with γ-irradiation. Cancer Lett *160*: 107-113, 2000.

4 Vesely J, Havlicek L, Strnad M *et al*: Inhibition of cyclin-dependent kinases by purine analogues. Eur J Biochem *224*: 771-786, 1994.

5 Shen SC, Huang TS, Jee SH and Kuo ML: Taxol-induced p34cdc2 kinase activation and apoptosis inhibited by 12-*O*-tetradecanoylphorbol-13-acetate in human breast MCF-7 carcinoma cells. Cell Growth Differ *9*: 23-29, 1998.

6 Papazisis KT, Geromichalos GD, Kouretas D, Dimitriadis KA and Kortsaris AH: CDK-inhibitor olomoucine inhibits cell death after exposure of cell lines to cytosine-arabinoside. Cancer Lett *138*: 221-226, 1999.

7 Park DS, Farinelli SE and Greene LA: Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons. J Biol Chem *271*: 8161-8169, 1996.

8 Park DS, Morris EJ, Stefanis L *et al*: Multiple pathways of neuronal death induced by DNA-damaging agents, NGF deprivation, and oxidative stress. J Neurosci *18*: 830-840, 1998.

9 Maas JW Jr, Horstmann S, Borasio GD, Anneser JMH, Shooter EM and Kahle PJ: Apoptosis of central and peripheral neurons can be prevented with cyclin-dependent kinase/mitogen-activated protein kinase inhibitors. J Neurochem *70*: 1401-1410, 1998.

10 Pulvertaft JV: Cytology of Burkitt's tumour (African lymphoma). Lancet *39*: 238-240, 1964.

11 Crissman HA: Cell cycle analysis by flow cytometry. *In*: Cell Growth and Apoptosis. A Practical Approach. Studzinski (ed.). New York: Oxford University Press Inc., pp. 22-23, 1995.

12 Crissman HA and Steinkamp JA: Rapid, simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. J Cell Biol *59*: 766-771, 1973.

13 Krishan A: Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol *66*: 188-193, 1975.

14 Van Dilla MA, Trujillo TT, Mullaney PF and Coulter JR: Cell microfluorometry: a method for rapid fluorescence measurement. Science *163*: 1213-1214, 1969.

15 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*: 248-254, 1976.

16 Martin SJ, Reutelingsperger CP, McGahon AJ *et al*: Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med *182*: 1545-1556, 1995.

17 Magaud JP, Sargent I and Mason DY: Detection of human white cell proliferative responses by immunoenzymatic measurement of bromodeoxyuridine uptake. J Immunol Methods *106*: 95-100, 1988.

18 Greenblatt MS, Bennett WP, Hollstein M and Harris CC: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res *54*: 4855-4878, 1994.

19 Brizuela L, Gyuris J and Mansuri M: Cyclin-dependent kinases and their regulators as potential targets for anticancer therapeutics. *In*: Principles of Molecular Oncology. Bronchud MH, Foote M, Peters WP, Robinson MO (eds.). New Jersey: Humana Press, pp. 197-236, 2000.

20 Boutis AL, Papazisis KT, Destouni E, Pistevou-Gompaki E, Papadopoulos L, Lambropoulos A, Boutis L and Kortsaris AH: CDK-inhibitor olomoucine and gamma-irradiation induced apoptosis and cell cycle arrest in Raji and K562 cell lines. Proc Amer Assoc Cancer Res *43*: 129, 2002 (Abstract 650).

21 Boulares AH, Zoltoski AJ, Contreras FJ, Yakovlev AG, Yoshihara K and Smulson ME: Regulation of DNAS1L3 endonuclease activity by poly(ADP-ribosyl)ation during etoposide-induced apoptosis. Role of poly(ADP-ribose) polymerase-1 cleavage in endonuclease activation. J Biol Chem *277*: 372-378, 2002.

22 Luciani MG, Oehlmann M and Blow JJ: Characterization of a novel ATR-dependent, Chk1-independent, intra-S-phase checkpoint that suppresses initiation of replication in Xenopus. J Cell Sci *117*: 6019-6030, 2004.

23 Enders GH and Maude SL: Traffic safety for the cell: Influence of cyclin-dependent kinase activity on genomic stability. Gene *371*: 1-6, 2006.

Received May 5, 2006
Accepted June 27, 2006