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Imbalance of Deoxyribonucleoside Triphosphates and DNA Double-strand Breaks in Mouse Mammary Tumor FM3A Cells Treated *in vitro* with an Antineoplastic Tropolone Derivative

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The mechanism by which α,α-bis(2-hydroxy-6-isopropyltropon-3-yl)-4-methoxytoluene (JCI-3661) kills mouse mammary tumor FM3A (F28-7) cells was studied. When the cells were exposed to the drug at 3.7 μM, the intracellular dNTP pool became imbalanced because of decreases in dGTP and dATP and an increase in dTTP. The pattern of the dNTP imbalance was the same as that caused by hydroxyurea. When JCI-3661 was added to the culture medium, mature DNA strands broke, giving fragments of 100–200 kilobase pairs long as found by orthogonal-field-alternation gel electrophoresis. DNA strand breaks, detected by this technique, were observed in the cells at 12 h after the addition. The beginning of cell death was observed at about 14 h (trypan blue staining) or at about 12 h (colony-forming ability) after cultivation Breaks in the single and double strands of DNA, as measured by alkaline and neutral filter elution assay, became evident 24 h after treatment with 3.7 μM JCI-3661. Comparison of the ratio of single- and double-strand breaks caused by JCI-3661 to that following radiation suggested that JCI-3661 broke only double strands. Cycloheximide inhibited both the breakage of double strands and the cell death caused by JCI-3661. JCI-3661 decreased DNA synthesis more than RNA or protein synthesis. The breaks in double strands of DNA were probably important in the cell death caused by JCI-3661.

Key words: DNA double-strand break — dNTP imbalance — Cell death — Anti-neoplastic drug — Tropolone

α,α-Bis(2-hydroxy-6-isopropyltropon-3-yl)-4-methoxytoluene (JCI-3661,[3] Fig. 1) is a bistropolone compound[1,2] that has an anti-neoplastic activity *in vivo* against murine P388 leukemia cells. This compound chelates metals strongly, so we suspected that its target might be ribonucleoside diphosphate reductase, and we studied its effects on the synthesis of nucleic acids and nucleotides.[3,4] This enzyme contains a pair of non-heme irons and a tyrosyl free radical as part of its primary structure,[5,6] and hydroxyurea, a radical scavenger, interacts with the tyrosyl free radical, thereby interfering with the enzyme action, which results in changes in the intracellular pools of deoxyribonucleoside triphosphates (dNTPs).[7] The treatment of mouse mammary tumor FM3A cells *in vitro* with 5-fluorodeoxyuridine (FrdUrd), deoxyadenosine, or 2-chlorodeoxyadenosine (2-CldAdo) imbalances cellular dNTP, followed by double-strand breaks in the mature DNA, and cell death.[8–11] Here, we measured the intracellular dNTP pool in FM3A cells treated with JCI-3661 and compared it with that of cells treated with hydroxyurea, an inhibitor of ribonucleoside diphosphate reductase. Hydroxyurea kills FM3A cells because it causes an imbalance in intracellular dNTP, which seems to break DNA double strands. The cytotoxicity of JCI-3661 may arise from the same mechanism.

We also measured DNA strand breaks in and the cell viability of FM3A cells treated with JCI-3661, and compared the results with those found for cells treated with hydroxyurea. The dNTP imbalance caused by JCI-3661 was followed by breaks in the double strands of DNA and later by cell death.

MATERIALS AND METHODS

Materials Hydroxyurea was purchased from Wako Pure Chemical Industries, Osaka. JCI-3661 was synthesized as previously described.[1,2] Mouse mammary tumor FM3A cells (wild type, F28-7) were the generous gift of Prof. T. Seno (National Institute of Genetics, Mishima).

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[3] The abbreviations used are: JCI-3661, α,α-bis(2-hydroxy-6-isopropyltropon-3-yl)-4-methoxytoluene; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; rNTP, ribonucleoside 5′-triphosphate; FrdUrd, 5-fluorodeoxyuridine; 2CldAdo, 2-chlorodeoxyadenosine; 2CldAdoTP, 2-chlorodeoxyadenosine 5′-triphosphate; FdUMP, 5-fluorodeoxyuridine 5′-monophosphate; CDP, cytidine 5′-diphosphate; PBS, phosphate-buffered saline; SSB, single-strand breaks; DSB, double-strand breaks; OFAGE, orthogonal-field-alternation gel electrophoresis; HPLC, high-pressure liquid chromatography.

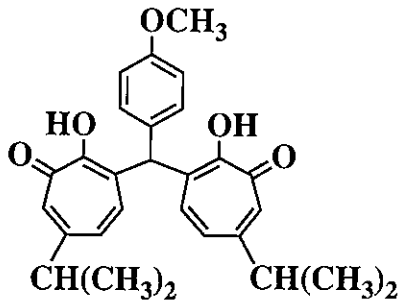

Fig. 1. Structure of JCI-3661.

Cell culture FM3A cells were grown at 37°C in a 5% CO_2 atmosphere in modified Eagle's minimun essential medium (ES medium, Nissui Pharmaceutical Co. Ltd., Tokyo) containing 2% fetal bovine serum inactivated with heat.[12] The cell cultures were maintained by passage twice weekly into fresh medium. Cells were counted with a cell counter (CC-108; Toa Medical Electronics Co., Kobe). For growth-inhibition studies, cells were seeded at about 5×10^4/ml, and treated with various concentrations of JCI-3661. Cells were counted 48 h after the start of incubation. The EC_{50} value is the concentration of JCI-3661 that reduced the growth rate of the cells by 50% of the control.[11] Cell viability during treatment with JCI-3661 was evaluated by trypan blue staining and by colony-forming ability.[8] The kinetics of the cell cycle were monitored as previously described.[11]

Preparation of cell extract JCI-3661 or hydroxyurea was added to a suspension culture of exponentially growing cells at the concentration of 0.19–9.3 μM or 10 mM, respectively. The drug was added when the cell density reached 2.5×10^5/ml. The volume of the cell suspension examined was 1000 ml. At certain times, a 50-ml sample of the suspension was removed from the culture bottle and centrifuged at 100g and 4°C for 10 min. The cells thus collected were washed twice with 25 ml of ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4\cdot H_2O$, and 1.4 mM KH_2PO_4, pH 7.3, containing 0.1% glucose), and suspended at 4°C in about 100 μl of the PBS. After the cells were counted, the suspension was transferred to a 1.5-ml Eppendorf tube (3810, Eppendorf, Hamburg, Germany), and cold 100% trichloroacetic acid was added to the suspension to the final concentration of 0.3 M. The mixture was vortexed, kept for 30 min at 4°C, and centrifuged. The acid supernatant was separated and to it was added 1.1 volume of cold Freon-amine solution (0.5 M tri-n-octylamine in 1,1,2-trichlorotrifluoroethane). The aqueous upper layer containing nucleotides was separated and assayed by high-pressure liquid chromatography (HPLC) for determination of dNTPs. The cell extracts were prepared as described previously.[13]

Assay of dNTPs in cell extract To 80 μl of cell extract in a 1.5-ml Eppendorf tube were added 20 μl of 20 mM deoxyguanosine and 20 μl of 0.2 M $NaIO_4$. After vortexing and centrifugation (15,600g at 4°C for 10 s) of the suspension, it was incubated at 37°C for 2 min. The tube was placed on ice, and then 2 μl of 1 M L-rhamnose and 30 μl of 4 M CH_3NH_2 (neutralized to pH 6.5 with H_3PO_4) were added. The suspension was mixed well and centrifuged at 15,600g and 4°C for 10 s. After incubation at 37°C for 30 min, the sample was cooled on ice.

Chromatography on a Partisil-10 SAX column (4.6× 250 mm, Whatman, Clifton, NJ) was done as described before.[13] HPLC was done with a Waters 6000A pump, a Waters 440 absorbance detector (at $A_{254\,nm}$), and a Hewlett-Packard 3390A integrator.

Ribonucleoside triphosphate (rNTP) pools were measured as described elsewhere.[13]

Detection of DNA strand breaks Cells were grown for 24 h in ES medium containing 1.2 μM [2-^{14}C]thymidine (2.4 GBq/mmol, DuPont/NEN Research Products, Wilmington, DE). Cultures were then incubated in the absence of the radiolabeled compound so that radioactivity would be incorporated into DNA of high molecular weight. Cells (density, 2×10^5/ml) with [^{14}C]DNA were incubated with JCI-3661 (3.7 μM) as described in "Cell Culture" above. At specified times, samples containing about 5×10^5 cells were rinsed with 10 ml of PBS and put onto polycarbonate filters (Nucleopore, 25 mm, pore size 2.0 μm) in Swinnex funnels (Millipore Corp., Bedford, MA) as described by Kohn *et al.*[14] Then 1.5 ml of lysis solution (50 mM Tris, 50 mM glycine, 25 mM Na_2EDTA, 2% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K, adjusted to pH 9.6 with NaOH) was added to the funnel and the mixture was left for 60 min at 25°C. The lysis solution was washed out by the addition of 3 ml of 20 mM EDTA (pH 10.0) to the funnel, and the mixture was allowed to flow into a scintillation vial. Next, the exit tube of the funnel assembly was connected to a tube leading to a peristatic pump. Then 20 ml of a tetrapropylammonium hydroxide-EDTA solution (pH 12.0, as described by Kohn *et al.*[15]) was poured into the syringe barrel, and elution was done at 25°C in the dark. Fractions were collected at every 90 min (flow rate, about 2 ml/1.5 h). The procedure used in this assay was essentially identical to that described by Kohn *et al.*[15, 16] The neutral elution was done as described previously[15, 16] with an eluent at pH 9.6 (50 mM Tris, 50 mM glycine, 25 mM Na_2EDTA, and 2% sodium dodecyl sulfate, adjusted to pH 9.6 with NaOH).

Cells were irradiated by a γ-ray irradiator with a cesium-137 source (1.85×10^4 GBq) at 4°C. The irra-

diated cells were analyzed immediately by the alkaline or neutral elution assay.

The ratio of single-strand breaks (SSB) to double-strand breaks (DSB) was calculated by use of the following equation[17,18]:

$$\frac{s}{d} = \left(\frac{k_{RS}}{k_{RD}}\right)\left(\frac{[SSD]}{[DSB]}\right) - 2,$$

where s/d is the ratio of the actual numbers of SSB and DSB. [SSB]/[DSB] is the ratio of gray equivalents (the dose of radiation causing an equivalent number of breaks) found by the alkaline elution for SSB and by neutral elution for DSB, k_{RS} is the frequency of SSB produced per unit of X-rays, as measured by the alkaline assay, and k_{RD} is the frequency of DSB produced per unit of X-rays. The actual ratio of SSB to DSB caused by X-rays, k_{RS}/k_{RD}, is between 10 and 40.[15,16] If a drug produced only DSB, s would equal to zero, and the measured [SSD]/[DSB] would therefore be 0.05 to 0.2. X-rays and γ-irradiation cause DNA strand breaks by the same mechanism. The numbers of DNA SSB and DSB were calculated from the elution profiles and expressed as gray equivalents as estimated from calibration curves.[14-18]

Incorporation of labeled thymidine into DNA For evaluation of the effects of JCI-3661 on thymidine incorporation into DNA, FM3A cells at a density of 2×10^5/ml were incubated at 37°C in a medium containing JCI-3661 (3.7 μM) and [^{3}H-*methyl*]thymidine (37 KBq/ml, 740 GBq/mmol, DuPont/NEN Research Products). At certain times, duplicate samples were removed and washed twice with cold/PBS. Trichloroacetic acid was added to the cell suspension at 4°C to give a final concentration of 10%. The mixture was kept for 15 min at 0°C and filtered on nitrocellulose filter disks (TM-2, Advantic, Tokyo). The disks were washed five times with 5 ml of cold 5% trichloroacetic acid. Disks were dried and dissolved in 0.8 ml of dimethyl sulfoxide, and the radioactivity was measured in Triton X-100 scintillation fluid.

For evaluation of the effects of JCI-3661 on protein and RNA synthesis, incubation was done in the same way, except that L-[4,5-^{3}H]leucine (37 KBq/ml, 185 GBq/mmol, DuPont/NEN Research Products) or [5-^{3}H]uridine (37 KBq/ml, 1.055×10^3 GBq/mmol, DuPont/NEN Research Products) was used in place of the labeled thymidine.

Orthogonal-field-alternation gel electrophoresis The apparatus used for orthogonal-field-alternation gel electrophoresis (OFAGE) was as described previously.[19] Agarose blocks containing intact chromosomal DNA from *Saccharomyces cerevisiae* BO133-3B were prepared as described elsewhere.[20] Agarose blocks containing DNA from FM3A cells growing asynchronously and treated with 3.7 μM JCI-3661 were prepared by a slight modification of the procedure of Schwartz and Cantor.[20] JCI-3661 was added when the cell density reached 2×10^5/ml. At certain times, cells were harvested and washed once with PBS at 4°C; the cells (about 2×10^6) were then placed in 0.6 ml of 0.6% low-melting agarose (Bio-Rad Laboratories, Richmond, CA). The cells were lysed in an aqueous solution of 0.5 M EDTA, pH 9.0, 1% lauroyl sarcosine, and 1 mg/ml proteinase K for 2 days at 50°C. Blocks of DNA were washed three times in 0.2 M EDTA, pH 8, before being stored at 4°C. Electrophoresis was performed as described by Carle and Olson[19] with 0.6×TBE (53 mM Tris, 53 mM boric acid, and 1.2 mM EDTA, pH 8.3) at 13°C for 18 h using linear gradient pulses of 50 to 100 s. The buffer was recirculated and changed at the midpoint of the run to improve the resolution of the DNA samples. The gel was stained with ethidium bromide.

Preparation of ribonucleotide reductase and assay of its activity About 5.0×10^8 FM3A cells were suspended in ice-cold extraction buffer (50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 10 mM dithiothreitol, and 2 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride) and homogenized by sonication. After centrifugation of the homogenate at 100,000g for 60 min at 4°C, the supernatant was removed and streptomycin was added to a final concentration of 0.5%. The mixture was centrifuged at 20,000g for 30 min. The supernatant was removed and ammonium sulfate added to it to 40% saturation (0.25 g/ml). The mixture was centrifuged at 20,000g for 30 min at 4°C and the precipitate obtained was dissolved in 50 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol, and 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride. This solution (the crude extract) was dialyzed against the above sulution and then stored at −80°C.[21]

Ribonucleotide reductase activity was measured by cytidine 5′-diphosphate (CDP) reduction. The assay mixture contained 10 mM Tris-HCl (pH 7.6), 4.2 mM magnesium acetate, 10 mM dithiothreitol, 5 mM NaF, 1.6 mM ATP, 2.5 mM CMP, crude extract, and 1.2 μM [5-^{3}H]CDP (814 GBq/mmol, DuPont/NEN Research Products). The assay was done as described before[22] except that the volume of the assay mixture was 40 μl and that the incubation was for 120 min at 37°C. The reaction was stopped by boiling of the mixture for 2 min. The mixture was then centrifuged for 5 min at 15,800g. A sample of the supernatant was incubated at 37°C for 2 h with 10 μl of snake venom (5 mg/ml, Sigma, St. Louis, MO) and then boiled for 2 min to stop the hydrolysis. Ten microliters of the mixture was spotted onto a Polygram CEL 300 PEI plate (Macherey-Nagel, Düren, Germany) pretreated with ammonium tetraborate and developed in a 1:1 mixture of 20 mM ammonium formate and ethanol. Spots of both nucleosides and deoxynucleosides

were removed from the plate and their radioactivities were determined in a toluene-based scintillation fluid by using a liquid scintillation counter.

RESULTS

The EC_{50} values for JCI-3661 and hydroxyurea towards FM3A cells were 0.57 μM and 60 μM, respectively. As determined by trypan blue exclusion, cell viability began to decrease at around 14 h of exposure to 3.7 μM JCI-3661. At 36 h, about 30% of the cells excluded trypan blue. The cell death caused by this drug was prevented by the addition of cycloheximide at 2 μg/ml (Fig. 2). The loss of colony-forming ability occurred at about 12 h (data not shown). When the cells were exposed to 3.7 μM JCI-3661 for 24 h, washed twice with fresh ES medium, incubated for 2 days in fresh ES medium, and then plated on 0.5% soft agar, no colony formation was observed. When the cells were exposed to 10 mM hydroxyurea for 24 h, 50% of the cells excluded trypan blue. The colony-forming ability was about 1%. This indicates that 10 mM hydroxyurea is equivalent to 3.7 μM JCI-3661 with respect to the cytotoxicity.

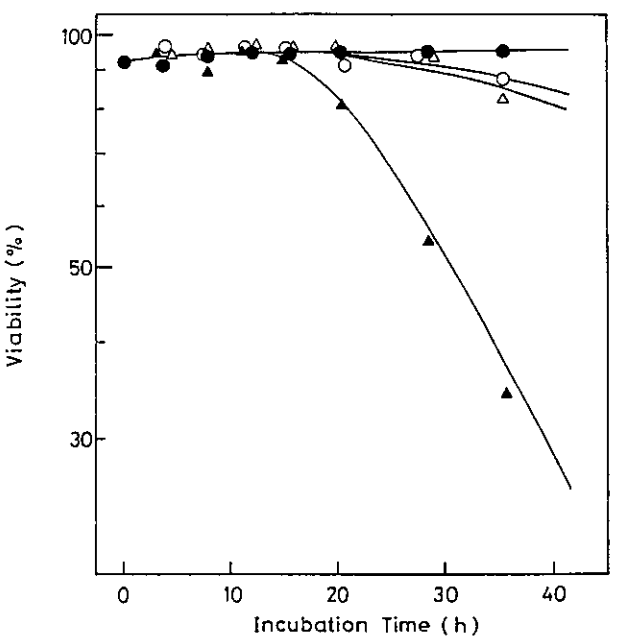

Fig. 2. Effect of cycloheximide on viability of FM3A cells treated with JCI-3661. Asynchronously growing FM3A cells were cultured in the presence or absence of 3.7 μM JCI-3661 with cycloheximide (2 μg/ml) or without. The viability of cells was evaluated by trypan blue exclusion. Incubation with JCI-3661 only (▲); with both JCI-3661 and cycloheximide (△); with cycloheximide only (○); and without JCI-3661 or cycloheximide (●).

Cycloheximide at 2 μl/ml prevented the cell death caused by the treatment with 10 mM hydroxyurea for 24 h (data not shown).

Figure 3 shows changes in the intracellular dNTP pools as a function of the time of treatment with JCI-3661 or hydroxyurea. Changes were significant. With JCI-3661, the dATP pool decreased to about 40% of the control after 4 h of incubation and to 10% after 12 h, and the dGTP pool size was below the limit of detection when assayed at 4 h. A large increase in dTTP and an insignificant increase in dCTP were observed at 4 h. The changes in the pools after treatment with JCI-3661 or hydroxyurea were similar. Intracellular rNTP pools did not change with JCI-3661 treatment (data not shown). To investigate the changes in the size of dNTP pools with various concentrations of JCI-3661, cells were incubated with 0.19–9.3 μM JCI-3661 at 37°C for 8 h (Fig. 4). The dGTP and dATP pools decreased as the concentration of JCI-3661 increased, whereas the dCTP and dTTP pools increased.

We investigated whether or not DNA strand breaks were caused by such treatment. DNA SSB and DSB, as measured by the alkaline and neutral filter elution, were detected at 24 h after treatment with JCI-3661 (Fig. 5). The number of SSB corresponded to that which would be obtained with γ-irradiation at 1.5 Gy. In contrast, the number of DSB corresponded to that would be obtained by γ-irradiation at 21.2 Gy. The ratio [SSB]/[DSB] was

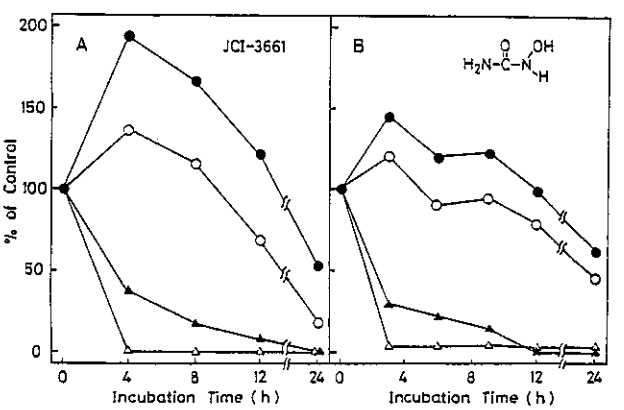

Fig. 3. Changes in the size of dNTP pools in FM3A cells treated with JCI-3661 or hydroxyurea. FM3A cells at the density of 2×10^5/ml were treated with 3.7 μM JCI-3661 (A) or 10 mM hydroxyurea (B). At the indicated times, 50-ml samples were removed and dNTP pools were measured as described in "Materials and Methods." ▲, dATP; △, dGTP; ○, dCTP; and ●, dTTP. Retention times of dNTPs were as follows (min): dATP, 11.7; dGTP, 19.6; dCTP, 7.5; dTTP, 8.6. The dNTP pool size at time zero was as follows (pmol/10^6 cells): dATP, 21.9; dGTP, 6.7; dCTP, 26.3; and dTTP, 40.9.

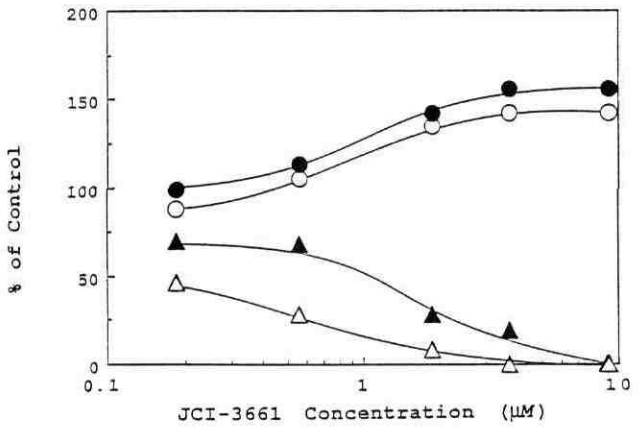

Fig. 4. Changes in size of dNTP pools in FM3A cells treated with various concentrations of JCI-3661 for 8 h. FM3A cells at the density of 2.0×10^5/ml were treated with various concentrations of JCI-3661. At 8 h after the addition of the drug, 50-ml portions were removed and dNTP pools were measured by HPLC as described in "Materials and Methods," ▲, dATP; △, dGTP; ○, dCTP; and ●, dTTP.

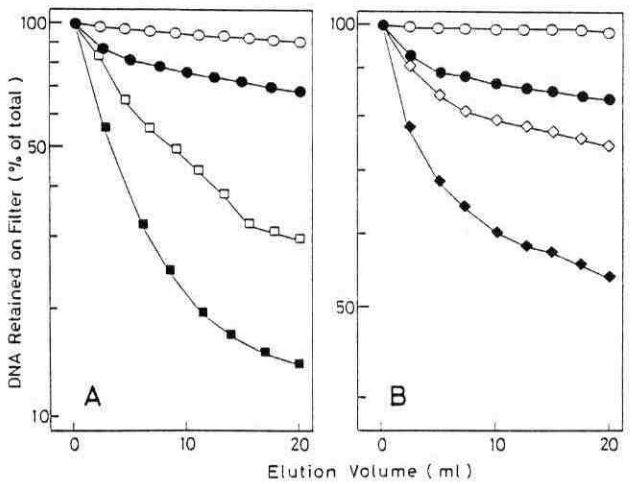

Fig. 5. Alkaline (A) and neutral (B) elution patterns of cells treated with JCI-3661. FM3A cells labeled with [^{14}C]thymidine were incubated with JCI-3661 (3.7 μM) for 24 h (●). For comparison, γ-irradiation was done: in A, exponentially growing cells were irradiated with 3 (□) or 6 (■) Gy, and in B, the cells were irradiated with 30 (◇) or 60 (◆) Gy. Control cells: ○.

0.071. At 30 h after the start of treatment with JCI-3661, the SSB and DSB were at 1.95 and 42.5 Gy equivalents, respectively (data not shown). These results suggest that the DSB were not the result of an accumulation of SSB,

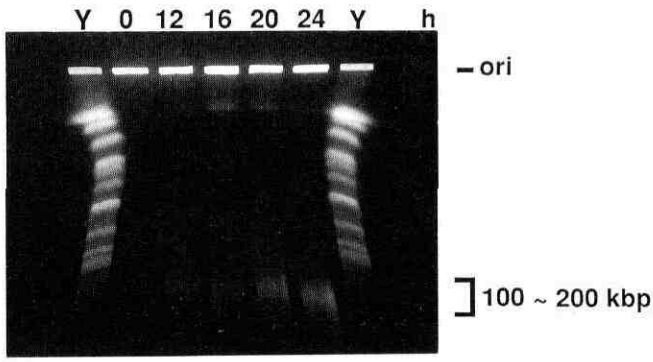

Fig. 6. OFAGE of breaks in DNA strands during JCI-3661 treatment. Agarose blocks containing cells treated with 3.7 μM JCI-3661 were prepared as described in "Materials and Methods." The times indicate the time of incubation with JCI-3661. The gel contains cell block preparations of *S. cerevisiae* (Y). Ori is origin.

but were formed by direct double-strand cuts. The DSB caused by 3.7 μM JCI-3661 were prevented by co-incubation with 2 μg/ml cycloheximide, which inhibits protein synthesis. DSB caused by JCI-3661 were directly examined by electrophoresis (Fig. 6). DNA of untreated cells was retained at the origin of the gel. During the exposure of FM3A cells to 3.7 μM JCI-3661 for 24 h, fragmented DNA bands appeared. In cells treated with JCI-3661, fragmented DNA bands were not observed during the first 10 h (data not shown). After 12 h of treatment, a band appeared near the region of 100–200 kilobase pairs (kbp), which is close to the postulated size of replication units in mammalian cells.[23] We used DNA from λ phage (48.5 kbp), T4 phage (166 kbp), herpes simplex virus type 1 (150 kbp), and yeast (260–2000 kbp) as size markers. The analysis of γ-irradiated FM3A cells (60 Gy) by OFAGE shown the formation of fragmental DNA at about 1000–2000 kbp, and there was no band in the region of 50–1000 kbp. The lethal dose of γ-ray for FM3A cells was 12.5 Gy (Y. Wataya, unpublished results). We tried to relate the cell death with the extent of the DNA fragmentation. However, the attempt was not successful, because the amounts of DNA in the OFAGE plate were not quantifiable using the ethidium bromide staining. Phage ϕX174 RFI DNA (supercoiled, double-stranded circular form) was incubated with JCI-3661 (10 μM) at 37°C and pH 7.4 in PBS (without glucose) for 3 h and electrophoresed in agarose as described previously.[24] DNA strand scission was not observed.

JCI-3661 inhibited the CDP reduction by ribonucleotide reductase from FM3A cells. At 3.7 μM, JCI-3661 inhibited CDP reduction to 37% of the control. As

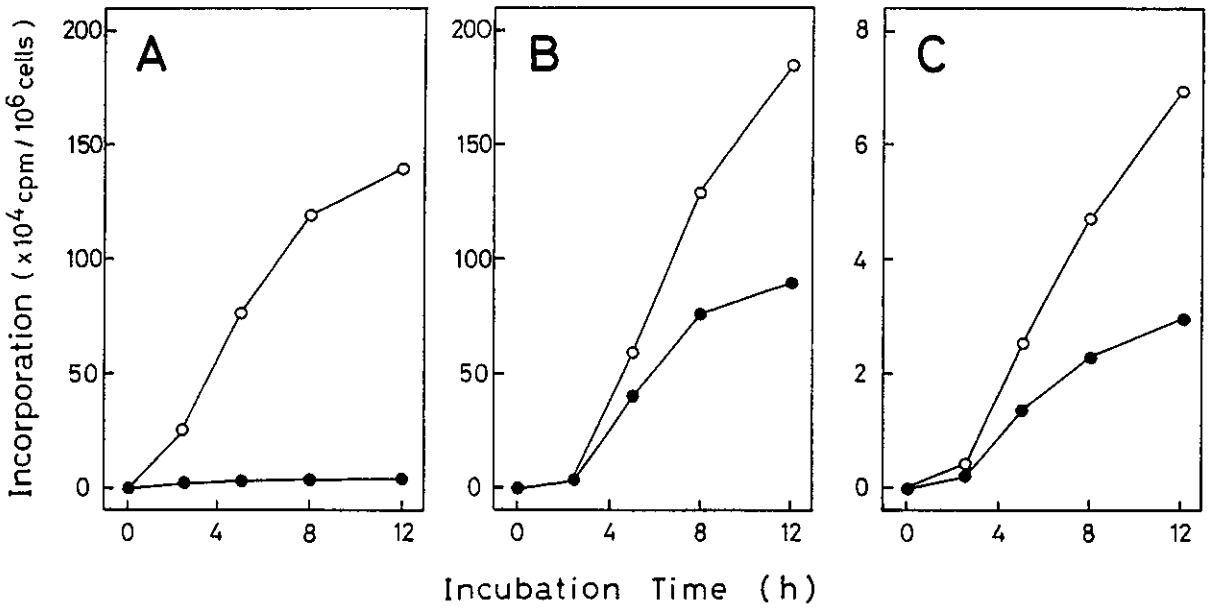

Fig. 7. Effect of JCI-3661 on the incorporation of the labeled precursors [^{3}H-*methyl*]thymidine, [5-^{3}H]uridine, and [4,5-^{3}H]leucine into DNA (A), RNA (B), and protein(C) of FM3A cells. Experiments were done as described in "Materials and Methods" with continuous exposure of the cells to of 3.7 μM JCI-3661 (●) or without the drug (○).

expected, hydroxyurea (at 40 mM), a known inhibitor of the reductase, inhibited 70% of the enzyme activity.

When FM3A cells in culture were exposed to 3.7 μM JCI-3661, [^{3}H-*methyl*]thymidine incorporation into DNA was inhibited; at 12 h, the incorporation was 3% of the control rate (Fig. 7A). At 12 h, the incorporations of [4,5-^{3}H]leucine into protein and [5-^{3}H]uridine into RNA were decreased by JCI-3661 to 48% and 43% of the control, respectively (Fig. 7B and C).

During exposure of FM3A cells to 3.7 μM JCI-3661 for up to 24 h, JCI-3661 caused a redistribution in the percentage of cells in the different phases of the cell cycle. The percentage of cells in the G_1 phase increased. At the start of incubation, 38% of cells were in the G_1 phase and 36% were in S phase. At 24 h, these figures were 57% and 36%, Analysis by computer simulation showed that most of the cells in G_1 phase treated with JCI-3661 were between the G_1 and S phases.

DISCUSSION

Incubation of FM3A cells with JCI-3661 caused intracellular dNTP pool imbalance, DNA double-strand breakage, and loss of cell viability. Treatment of FM3A cells with deoxyadenosine causes dNTP imbalance, followed by DNA DSB and cell death.[9] It is generally accepted that unrepaired DNA DSB are lethal for cells.[25] DNA strand breaks, giving fragments of 100–200 kbp as found by electrophoresis, were observed in the treated cells about 12 h after the addition. The cells lost viability at about 14 h (trypan blue staining as shown in Fig. 2) or at 12 h (colony-forming ability). These results lead us to propose that DNA DSB play an important role in the mechanism of JCI-3661-mediated cell death. DSB seem to be an important factor in the cell death mediated by FrdUrd, deoxyadenosine, or 2-CldAdo, and intracellular dNTP imbalance may trigger the events.[8–11] The cytotoxicities of JCI-3661, FrdUrd, deoxyadenosine, and 2-CldAdo are similar, although the patterns of the resulting dNTP imbalance are different. The depletion of dTTP as a result of the inhibition of thymidylate synthase by FdUMP, a metabolite of FrdUrd,[8] and the increases of dATP and 2CldAdoTP that can result from the intracellular conversions of deoxyadenosine[9] and 2CldAdo[10, 11] may interfere with the regulation of ribonucleotide reductase, which in turn could disturb the balance of dNTP pool. Since JCI-3661 inhibited ribonucleotide reductase prepared from FM3A cells and, in addition, JCI-3661 and hydroxyurea gave rise to similar dNTP imbalance,we consider that JCI-3661 may act by a mechanism similar to that of hydroxyurea, i.e., by inhibiting ribonucleotide reductase. Coyle and Strauss[26] have shown that incubation of HEp-2 cells with hydroxyurea leads to an intracellular accumulation of DNA fragments. In cultured Ehrlich ascites tumor cells, hydroxyurea causes strand breaks in mature DNA,

paralleled by cell death.[27] As dNTP pools are very small, 1/1000 of the total amount of deoxyribonucleotide residues in DNA,[28] depletion of any dNTP would greatly hinder DNA synthesis.[29] The inhibition of DNA synthesis by JCI-3661 is probably the result of the inhibition of dNTP synthesis, i.e., depletion of dATP and dGTP. JCI-3661 inhibits DNA synthesis in FM3A cells more than RNA or protein synthesis, as is the case for 2-CldAdo.[11] Decrease in RNA and protein synthesis by JCI-3661 may be due to the blockage of the cell cycle at the boundary between G_1 and S phases.

Our hypothesis about the mechanism of cell death caused by JCI-3661 is similar to the hypotheses made in studies of the FrdUrd, deoxyadenosine, and 2-CldAdo.[8-11] The dNTP pool imbalance could signal the activation of an endonuclease or the gene. Evidence for this view is that an endonuclease, which is active in 10 m*M* Hepes buffer (pH 7.4) containing 5 m*M* EDTA (EDTA does not inhibit the enzyme reaction) and is inactive in the buffer containing 10 m*M* $MgCl_2$ or 10 m*M* $CaCl_2$, and which can cause DNA DSB, was found in the lysate of FM3A cells treated with JCI-3661, but not in the untreated cells (unpublished results; the details will be published elsewhere). The endonuclease induced by the drugs would then attack DNA, causing DSB, and an accumulation of DNA DSB would cause cell death. We did not observe cell damage or cell death before the DNA DSB took place. In preliminary experiments, thymidine and deoxyadenosine were found at the 5′-termini at the ends of the DNA fragments produced by JCI-3661 treatment (unpublished results).

Cycloheximide, an inhibitor of protein synthesis,[30] inhibits thymineless death[31] and the death of FM3A cells caused by FrdUrd, deoxyadenosine, and 2-CldAdo.[8-11] We examined its effect on the DSB caused by JCI-3661 and cell death, and found that it can prevent both. It therefore seems that JCI-3661-induced cell death requires protein biosynthesis. JCI-3661 did not cleave ϕX174 RFI DNA directly, but we could not rule out the possibility that inhibition of metabolic enzymes that act on JCI-3661 results in free radicals that interact with the DNA. However, DNA DSB were not found in a mixture of JCI-3661 with the lysate of untreated FM3A cells, because JCI-3661 was inactivated by metals in the cell extracts (unpublished results).

Hydroxyurea is generally regarded as an S-phase specific agent and its cytotoxicity has been said to arise from the inhibition of DNA replication.[32-34] Our study with flow cytometry showed that JCI-3661 brought about accumulation of cells at the boundary between the G_1 and S phases. Events crucial for the regulation of cell growth seem to occur in the G_1 phase.[35] There seems to be a commitment point in G_1 phase; once the biochemical event associated with this point has occurred, a cell is irreversibly committed to initiate DNA synthesis and undergo cell division,[35] so cells treated with 2-CldAdo, FrdUrd or thymineless death probably pass through the commitment point, and the mechanism causing cell death from dNTP imbalance goes into action.

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