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We would like to submit the manuscript “The Effect of Colcemid on the Repair of UVC-Induced DNA Damages in Chinese Hamster Ovary Cells” for publication in the Journal of DNA REPAIR. This manuscript is submitted under the consent of all its authors and is not considered else where besides this Journal. We report in this paper that colcemid had inhibitory effect on the repair of DNA damages induced by UV probably by hindering gap-filling or the steps after oligonucleotide excision during nucleotide excision repair. We value your response and thank you for your attention.
Best wishes,

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The Effect of Colcemid on the Repair of UVC-Induced DNA Damages in Chinese Hamster Ovary Cells

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Abstract

In our previous study, we found that colcemid, an inhibitor of mitotic spindle, promotes UVC-induced apoptosis in Chinese hamster ovary cells (CHO-K1). A brief treatment of colcemid on cells after UV irradiation could synergistically reduce the cell proliferation. In this study, colcemid was found to hinder the restoration of genomic integrity following UV irradiation based on results of the nucleoid size analysis and the comet assay. On the other hand, according to an enzyme-linked immunosorbent assay, colcemid did not affect the excision of [6-4] photoproducts or cyclobutane pyrimidine dimers induced by UV irradiation. Thus, we hypothesize that colcemid probably affected the step(s) after the oligonucleotide excision i.e. gap-filling and ligation during nucleotide excision repair pathways. This idea is consistent with the observation that colcemid promoted the cell death induced by UV or cisplatin but not by X-ray.

1. **Introduction**

When the genetic stability of mammalian cells is challenged by various environmental assaults such as ultraviolet light (UV), the DNA repair systems need to be turned on to counteract the deleterious effects of DNA lesions. Nucleotide excision repair is a mechanism to remove the DNA lesions induced by shortwave UV radiation (i.e. UVB or UVC) and by numerous chemicals including chemotherapeutic agents such as cisplatin. Two major classes of DNA lesions induced by UV are cyclobutane pyrimidine dimers (CPD) and [6-4] photoproducts (6-4PP). The pathway of nucleotide excision repair of mammalian cells has been known in quite detail[1,2]. In brief, the DNA lesions are identified by DDB (XPE) or installed RNA polymerase II, in non-transcribed or transcribed region, respectively. Then, the general transcription factor complex TFIIH, containing the XPB and XPD helicases, mediates strand separation at the site of the damage. The damage-containing sequences are removed by endonucleases XPG and ERCC1-XPF complex. Finally, the gaps created by the excision step are filled by PCNA dependent DNA polymerases and the ends are sealed by ligase I.

Genomic assault by evoking checkpoint pathway may arrest cell cycle progression for DNA repair or lead to apoptosis [3,4]. In our previous study, we found that the Chinese hamster ovary cells (CHO.K1) after UVC-irradiation treatment failed

to arrest at G1 and exhibited a certain degree of apoptosis [5]. This UV-induced apoptosis was exacerbated if the UV irradiated cells were cultured in growth medium containing colcemid, an inhibitor of mitotic spindle [5,6]. The similar if not identical effect of colcemid on DNA damage-induced apoptosis was also reported in other situation where colcemid was found to accelerate the DNA-damage induced apoptosis by actinomycin [7]. Although the potential of this colcemid effect in application to chemotherapy is high, the underlying mechanism of the colcemid effect remained unclear.

In this report, we examined if the enhancement effect of colcemid on UV induced-cell death could be linked to the colcemid effect on repair of DNA damage. Results of the study suggest that although colcemid did not affect the excision of 64 PP and CPD, it prevented the restoration of genomic integrity following UV irradiation. We hypothesize that colcemid may hinder the step(s) after removal of damaged oligonucleotides during nucleotide excision repair.

2. Materials and methods

2.1 Cell cultures. The CHO-K1 cells of a Chinese hamster ovary cell line were originally obtained from the American Type Culture Collection (Manassas, VA) and were cultured in McCoy's 5A medium (Gibco BRL, Gaithersburg, MD) supplemented

with 10% fetal calf serum, 0.22% sodium bicarbonate (pH 7.5), penicillin (100 U/ml), streptomycin (100 μg/ml), 0.03% glutamine. Cells were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2. XRS-6 cells, the X-ray and UV sensitive strain of CHO-K1 cells, were provided by Dr. W. G. Chou (Department of Life Science, National Tsing-Hua University) and cultured in the similar conditions as CHO-K1 cells.

*2.2 UV- irradiation treatmen*t. The procedures for UV irradiation were the same as those described previously [6]. Briefly, cells in the dish were washed with phosphate-buffered saline (PBS), uncovered and exposed to UV light (254 nm) in the marked area of the tissue culture hood that had been pre-calibrated for the required dose. Doses of UV- irradiation were calibrated with a UV radiometer (UVP, San Gabriel, CA).

2.3 Cell growth analysis. Same numbers of CHO-K1 cells were seeded in wells of 6-well plates for 1-2 days (to reach about 50 % confluence) before treatment of UV-irradiation. After treatment, the cells were cultured in media with or without colcemid (50 ng/ml) for 6 h and then in normal media for another 30 h before MTT cell proliferation assay or direct cell counts. For cell counts, the cells were harvested by trypsinization and the cell suspension obtained from each 60 mm dish was diluted

to 8 ml with PBS. Aliquots of 0.5 ml of the diluted cell suspension were mixed with 9.5 ml isotonic diluent (Hematronix, Benicia, CA) for cell count with the machine Cell-Dyn-300 (VetLab, Miami, FL).

2.4 *Nucleoid size analysis*. The method was used since nucleoids with damaged DNA are larger in size than those with intact DNA. The nucleoid size analysis based on forward light scatter of particles was performed according to the procedures described previously [8]. Briefly, cells were harvested from culture dishes by trypsinization and were lysed in a high salt buffer (2M NaCl, 10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100) to release the DNA in the nucleoids. The nucleoids were stained with ethidium bromide (10 μg/ml or the indicated concentration) for at least 15 min at room temperature, and the forward scatter signals of the nucleoids were collected with a flow cytometer (Becton Deckinson FACScan, Mountain View, CA).

2.5 Comet assay. The comet assay described previously [9] was adopted with some modifications. After UV and/or other treatments, cells (~2X10^4 cells) were harvested by trysinization and resuspended in 10 μl of 1 X PBS and then mixed with 1% low melting agarose (LMA, 100 μl per 35-mm dish culture). The mixture was spread on a slide precoated with poly-L-lysine and 1% LMA. The agarose-spread was allowed to

solidify on ice for 5 min before the third layer of 1% LMA (100 μl) was applied. Slides were dipped in a cold lysis solution (2.5 M NaCl , 100 mM EDTA, 10 mM Tris pH 10, 1% Triton X-100, 10 % DMSO) at 4^{o}C for at least 1 h. The slides were transferred to an electrophoresis box containing an alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13) and kept for 20 min at 4 ^{o}C. A current of 25 V (300 mA) was applied for 25 min. After electrophoresis, the slides were neutralized in 0.4 M Tris (pH 7.5) for 5 min and stained with PI (50 μg/ml). Nucleoids were examined with a confocal fluorescence microscope. A total of 50 comets taken randomly from five layers (ten comets per layer) of each slide were scored. According to the tail fluorescence intensity, tail length and nucleoid integrity, comets were classified and given score as undamaged cells (0), low to middle damaged cells (1-3), and highly damaged cells (4) as described previously [10].

2.6 ELISA of UV-induced DNA damage. CHO-K1 cells were exposed UV-irradiated (25 J/m^2) and recovered in media with or without colcemid (50 ng/ml). After 6 h, the growth media of all cultures were changed to the normal medium (i.e. without colcemid). The cells were harvested by trypsinization at indicated intervals following UV-irradiation. Genomic DNA of cells was purified using a Genomic DNA extraction kit (Qiagen, Crawley, UK). 64 PP and CPD were quantitated by an enzyme-linked

immunosorbent assay (ELISA) using 64 M-2 and TDM-2 monoclonal antibodies, respectively [11]. The procedures of ELISA for determining CPD from genomic DNA [12] were followed. In brief, 96-well polyvinylchloride flat bottom microtiter plates, precoated with 0.003% protamine sulfate, were coated with sample DNA. Equal amounts of DNA (200 ng or 10 ng per well for 64PP and CPD, respectively) calculated from the absorbance at 260 nm, were coated in the wells. The binding of monoclonal antibodies to photolesions in immobilized-DNA in wells (in quadruplicate) was detected with biotinylated $F(ab')_2$ fragment of goat anti-mouse IgG and then with streptavidin peroxidase. The absorbance of colored products derived from o-phenylene diamine was measured at 492 nm by Titertek Multiskan Plus MK (Labsystems, Helsinki, Finland).

2.7 Trypan blue exclusion analysis. Viable cell numbers were determined by trypan blue exclusion analysis [13]. The cells were harvested at the indicated time by trypsinization. Aliquots (100 μl) of cell suspension were mixed with equal volume of 0.4 % (W/V) trypan blue solution at room temperature for 5 minutes. Twenty μl of the cell mixture was placed on the hemocytometer immediately and observed under low power microscope. The number of stained cells and the percentage of viability were then determined.

3. Results

3.1 Colcemid acted synergistically with UV to reduce the cell growth

As mentioned earlier, colcemid enhances the UV-induced apoptosis. The effect of colcemid was recapitulated here by examining the effect of colcemid on cell proliferation of UV-irradiated cells as shown in Fig 1. The single treatments of colcemid and UV-irradiation caused cell growth inhibition by a negligible percent and 50%, respectively. However, the combined treatment (i.e. UV + colcemid) produced a significantly more inhibition (about 70%). In this experiment, treatment of colcemid was done after UV irradiation and only in a brief period i.e. 6 h as compare to 34 h of total incubation period following UV irradiation. It was noted that although colcemid caused mitotic arrest in control cells (see the increase of rounded cells at 6h, Fig 1B, top panel), it did not exhibit similar effect in UV-irradiated cells (Fig 1B, lower panel). These indicate that the enhancement of colcemid on growth inhibition of UV-irradiated cells was not due to mitotic arrest. We suspected that colcemid might affect the repair of DNA lesions.

3.2 Colcemid hindered the restoration of cellular DNA integrity after UV irradiation

To know if colcemid might affect DNA repair, we performed the nucleoid size analysis [8]. The nucleoids with damaged DNA are larger size than those with intact

DNA, and the nucleoid size can be measured by flowcytometer as forward light scatter of cell particles [14]. As a control experiment, the average nucleoid size of UV-irradiated cells was monitored for a period time (Fig 2A), and we found that the nucleoid size transiently increased but returned to initial value within 8 h. However, XRS, an X-ray and UV sensitive cell line derived from the CHO-K1 failed to produce similar result. We then studied the effect of colcemid on DNA repair and summarized the results of the repeated experiments in Fig 2B. A brief treatment of colcemid alone did not significantly alter nucleoid size (compared lanes 1 and 2, Fig 2B). However, the treatment of colcemid after UV irradiation hindered the restoration of the nucleoid sizes of UV irradiated cells to the initial value (compared lanes 1,3 and 5, Fig 2B). Similarly, a treatment of aphidicolin, a DNA synthesis/repair inhibitor prevented the UV-irradiated cells from returning to initial nucleoid size (lane 4 of Fig 2B).

In addition to nucleoid size analysis, the comet assay was performed for studying the effect of colcemid on DNA breaks of UV-irradiated cells. In the control experiment for this study, we found that DNA breaks increased temporarily following the UV irradiation and returned to initial level at 8 h (data not shown). Furthermore, Consistent with the previous results from nucleoid size assay, the presence of colcemid, prohibited the repair of DNA breaks. As shown in Fig 3, the level of DNA

breaks remained high in cells treated with colcemid or aphidicolin at 8 h following UV irradiation as compared to those of the control cells or cells received UV treatment only (compared lanes 1-3 and 4,5, Fig 3).

3.3 Colcemid did not inhibit the excision of UV-induced DNA damages

To see if colcemid affected the excision of UV-induced DNA damage, we performed ELISA of 64PP and CPD in genomic DNA of UV-irradiated cells at a period of time (0-8 h) after UV irradiation. The results shown in Fig 4 indicate that colcemid had no effect on the excision of 64PP and CPD. By 8 h following irradiation, most of 64PP (≥ 80%) had been removed by the nucleotide excision repair mechanism in cells. In contrast, only a few fraction of CPD was excised. This is consistent with the previous reports that rodent cells are ineffective in repairing CPD.

3.4 Colcemid enhanced the cell growth inhibition (or cell death) by UV or cisplatin but not X-ray

To know if the effect of colcemid involved nucleotide excision repair mechanism, we examined the effect of colcemid on cell growth of cells treated with cisplatin. Cisplatin, known an anticancer drug, causes DNA damages similar to those by UV-irradiation. The drug triggers cells the nucleotide excision mechanism for DNA

repair. In this experiment, we also studied the effect of colcemid on X-ray irradiated cells as a comparison. X-ray causes DNA strand breaks and involves repair mechanisms different from nucleotide excision repair. For this study, the dose of each specific treatment that causes about 50% growth inhibition was used. We found that 25 J/m^2 of UV, 16 Gy of X-ray and 12.5 μM cisplatin, respectively caused 50% of growth inhibition (data not shown). The results of this study shown in Fig 5A indicate that colcemid enhanced the growth-inhibition by UV-irradiation or cisplatin but not X-ray irradiation (see lanes 3-8, Fig 5A). We also examined the effect of colcemid on cell death rates due to the treatments. The results were shown in Fig 5B. Again, the cell death rates caused by UV or cisplatin but not X-ray was enhanced by colcemid.

4. Discussion

In our previous study, we found that UV induced apoptosis in CHO-K1 cells and the cell death was enhanced by colcemid [5,6]. In this study, we linked this effect of colcemid with repair of UV-induced DNA damages.

First, we found a brief treatment of colcemid had a synergistic effect on growth inhibition by UV (Fig 1A) and this effect was not due to mitotic arrest (Fig 1B). As reported previously by others, UV-irradiation can delay cell progression of S phase in the hamster cells [14], and this delay was likely to put off the mitotic arrest by

colcemid. Then, we found that a brief treatment of colcemid at low concentration could significantly hinder the restoration of nucleoid size and repair of DNA breaks in cells following UV irradiation (Fig 2 and 3). However, when the excision of UV-induced DNA lesions was examined by ELISA with specific antibodies to 64 PP or CPD, we found that colcemid had no effect on nucleotide excision (Fig 4). Taking together the results and the current model of nucleotide excision repair mechanism [1,2], we hypothesize that colcemid, similar to aphidicolin, inhibited the filling of the gaps created by the excision step (see Fig 6 for illustration). Colcemid as a microtubules inhibitor may somehow affect the recruitment of components of nucleotide excision repair mechanism such as DNA polymerase δ and ε, PCNA, RFC to fill up the gaps or DNA ligase I to seal the ends. This model is consistent with the finding that colcemid exacerbated cell growth inhibition (or cell death) incurred by UV or cisplatin but not by X-ray (Fig 5). The method of UV micro-irradiation developed by Mori et al [16, 17] can be used to test if colcemid affected the recruitment of DNA repair proteins. In addition, comet assay in a modified scheme can be used to see if the gaps persisted in UV–irradiated cells when colcemid was present.

In conclusion, we found that colcemid interfered the repair of UV-induced DNA damages probably at the steps after nucleotide excision. This interference may be

exploited for cancer chemotherapy involving nucleotide excision repair such as cisplatin.

Acknowledgements

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Figure legends

Fig 1. (A) Colcemid enhanced the growth inhibition caused by UV irradiation. About 75000 cells from the aliquot of a single cell suspension were seeded to each well of 6-well plates and incubated for 1-2 days before receiving UV irradiation (25 J/m^2). After UV irradiation, cells were incubated in medium with or without colcemid (50 ng/ml) for 6 h and for another 28 h in plain medium before MTT cell proliferation assay. Symbols: control, cells were cultured in the same conditions except received no UV or colcemid treatments; colcemid, cells received colcemid treatment only; UV, cells received UV irradiation only; UV+colcemid, cells were UV irradiated and then colcemid-treated. The % of relative viability of Y axis was calculated in basis on control. (B) microscopic examination of cells to observe the colcemid effect. Top panel, cells received colcemid at 1 h (left) and the same cells at 6 h later (right). Bottom, cells received colcemid after UV irradiation at 1 h (left) and 6 h later (right).

Fig 2. Nucleoid size analysis. (A) The parental CHO-K1 cells but not the UV hypersensitive strain, XRS-6 exhibited the restoration of nucleoid size at 8 h after UV irradiation. Cells were UV-irradiated at 8 J/m^2 and harvested for nucleoid size analysis at the indicated time after irradiation. Bars represent S.E. of at least three individual experiments. (B) Colcemid hindered the restoration of nucleoid size of CHO-K1 cells

at 8 h after UV irradiation. The experimental conditions were similar to those described in (A) except some cells (lanes 4 and 5) were treated with colcemid (0.05 μg/ml) or aphidicolin (15 μM), respectively after UV irradiation. Data represent the mean values ± standard errors from at least three individual experiments. Significant differences from the control (i.e. UV alone) are marked (*, P< 0.01, t-test).

Fig 3. Comet assay. Colcemid hindered the repair of CHO-K1 cells for DNA damage due to UV irradiation. Similar to those described in Fig 2, the CHO-K1 cells were UV irradiated (25 J/m^2) and then incubated with or without colcemid (0.05 μg/ml) or aphidicolin (15 μM) for 8 h before comet assay. Data represent the mean values ± standard errors from at least three individual experiments. Significant differences from the control (i.e. UV alone) are marked (*, P< 0.01, t-test).

Fig 4. Determination of 6-4PP and CPD after UV irradiation by using an ELISA. Cells were UV-irradiated at 25 J/ m^2 and then incubated in growth medium with or without colcemid. At the indicated period after irradiation, cells were harvested for genomic DNA isolation and subsequent ELISA for 6-4 PP (panel A) and CPD (panel B). The ELISA data were converted to % of total 6-4 PP or CPD remained relative to zero hour after irradiation and referred to the UV-dose and DNA damage induction

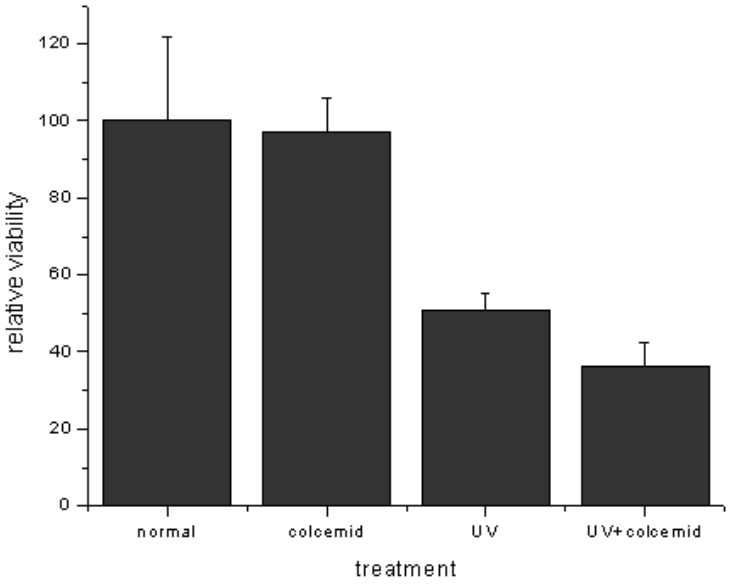
curve (data not shown). Data shown are mean values ± standard errors from at least 4 individual experiments.

Fig 5. (A) Colcemid synergistically inhibited cell growth with UV irradiation or cisplatin but not X-ray irradiation. The cells were grown in 60 mm dishes and were 50 % confluent before being treated with UV-irradiated (25 J/m^2; lanes 3 and 4) or X-ray irradiation (16 Gy; lanes 5 and 6) or cisplatin (12.5 μM; lanes 7 and 8). The cells were recovered in media with or without colcemid (0.05 μg/ml) for 6 h and then continually grown in media without colcemid for anther 30 h. Then, the cells were trypsinzed and the cell numbers were counted. Data were presented as % of cell counts relative to the control that received no particular treatments. (B) CHO.K1 cells were treated as described above. At 24 h after treatment, cells were harvested for trypan blue exclusion analysis. The numbers of stained cells of total more than 300 cells were counted and the percents of dead cells were determined. Significant differences from the controls (i.e. lanes 3, 5 and 7) are marked (*, $P< 0.02$, t-test). Data shown are mean values ± standard errors from at least three individual experiments.

Fig 6. Model of nucleotide excision repair and the plausible process affected by

colcemid. Colcemid did not affect recognition and excision of DNA damage, instead, it might affect the gap-filling or ligation steps.

Fig 1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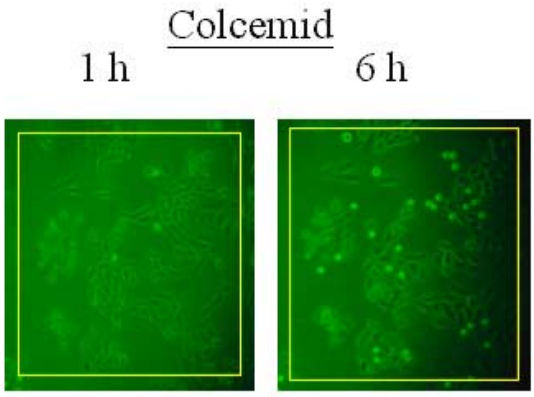
100
80
60
40
20
0
normal
colcemid
UV
UV+colcemid
treatment


Colcemid
1 h
6 h

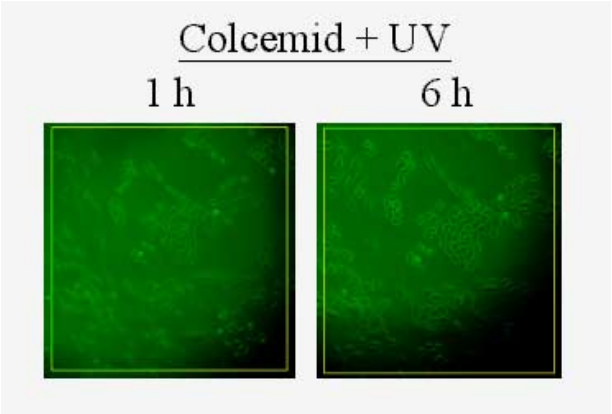
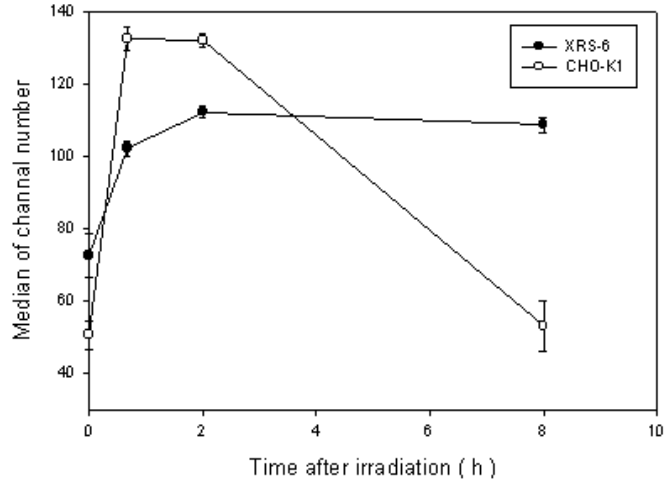

Colcemid + UV
1 h
6 h

Fig 2 (A)


XRS-6
CHO-K1
Median of channal number
Time after irradiation (h)
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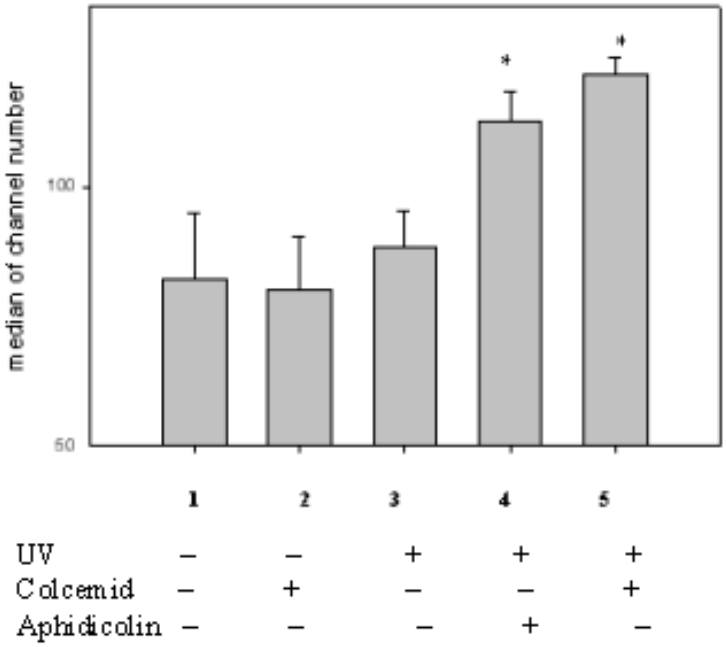
120
100
80
60
40
0
2
4
6
8
10

Fig 2(B)


median of channel number
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50
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*
1
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4
5
UV – – + + +
Colcemid – + – – +
Aphidicolin – – – + –

Fig 3

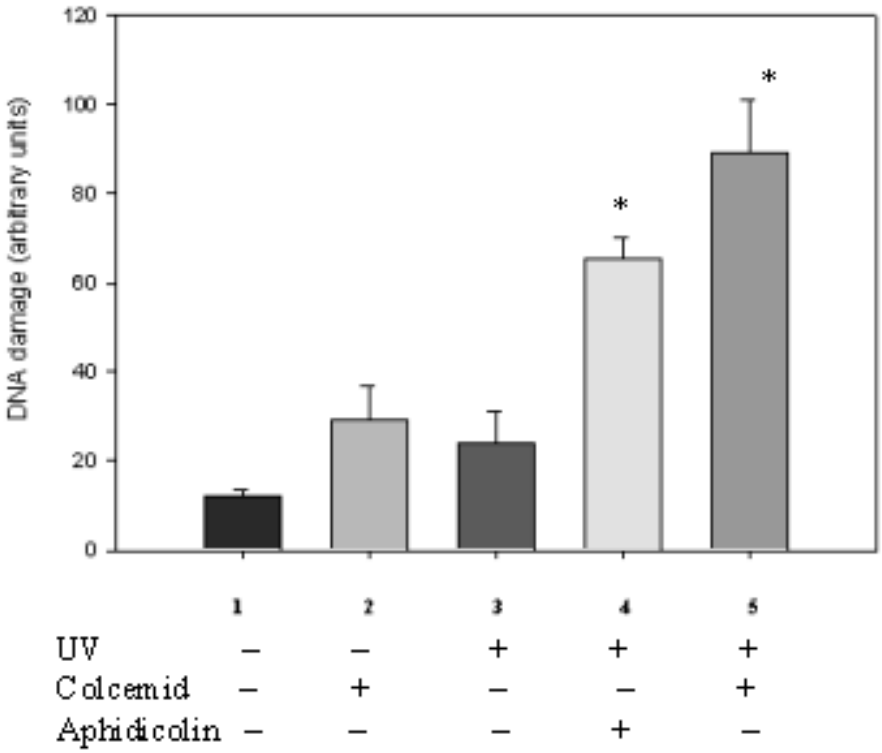
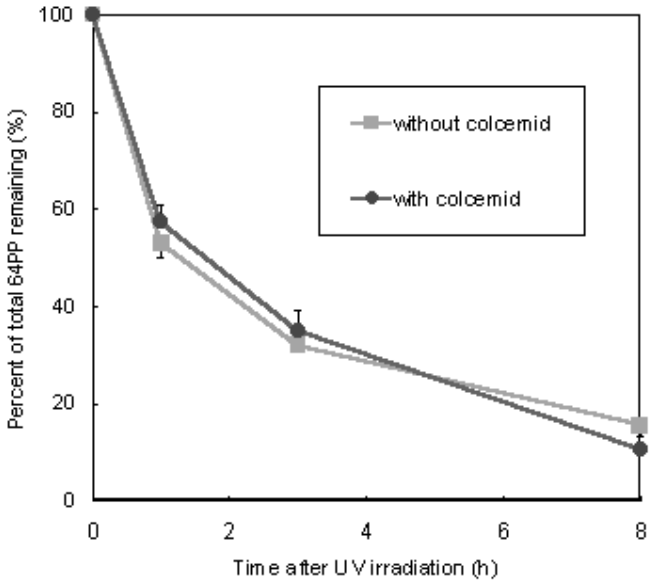

DNA damage (arbitrary units)
120
100
80
60
40
20
0
*
*
1
2
3
4
5
UV – – + + +
Colcemid – + – – +
Aphidicolin – – – + –

Fig 4

(A)


100
80
60
40
20
0
Percent of total 64PP remaining (%)
without colcemid
with colcemid
0
2
4
6
8
Time after UV irradiation (h)

(B)

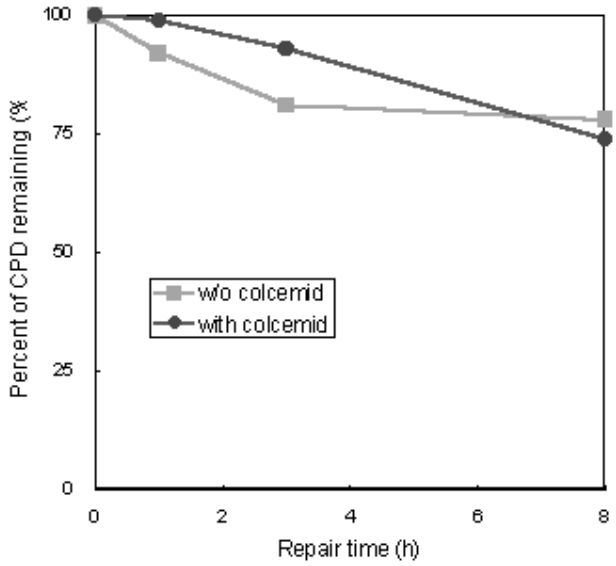
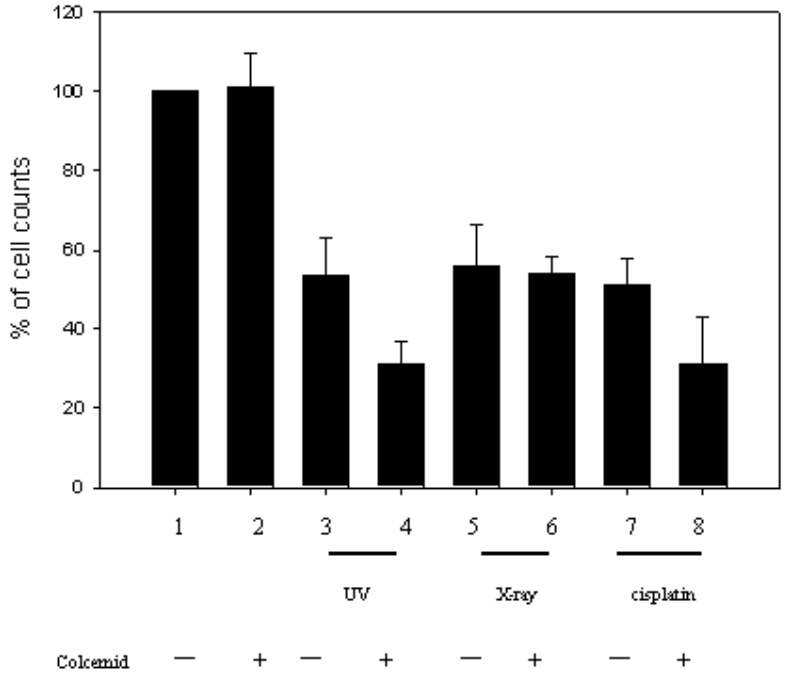

100
75
50
25
0
Percent of CPD remaining (%
w/o colcemid
with colcemid
0
2
4
6
8
Repair time (h)

Fig 5

(A)


% of cell counts
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
UV
Xray
cisplatin
Colcemid
—
+
—
+
—
+
—
+

(B)

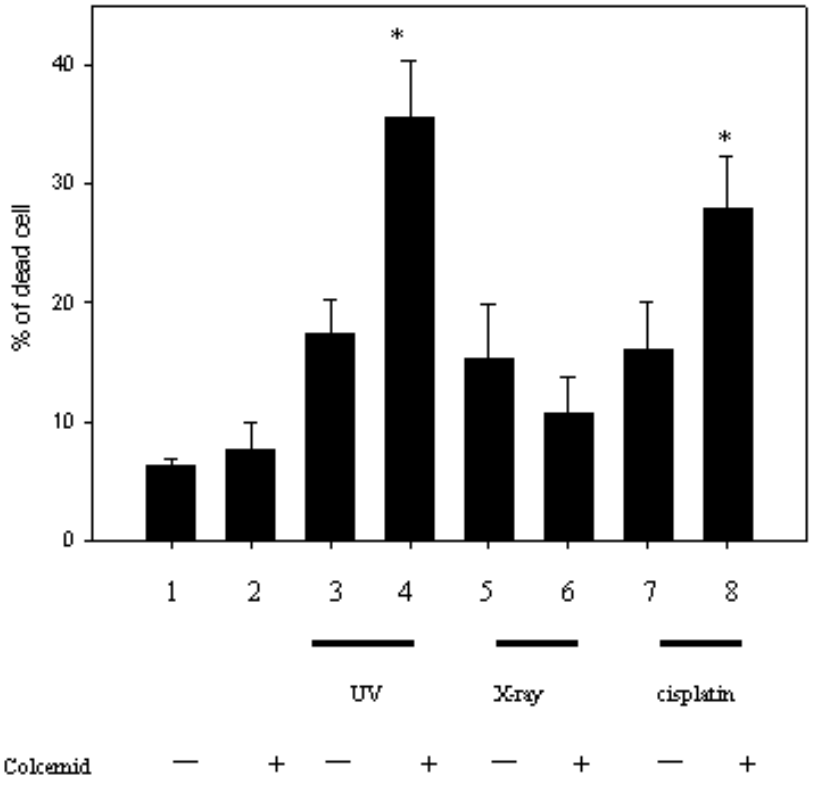
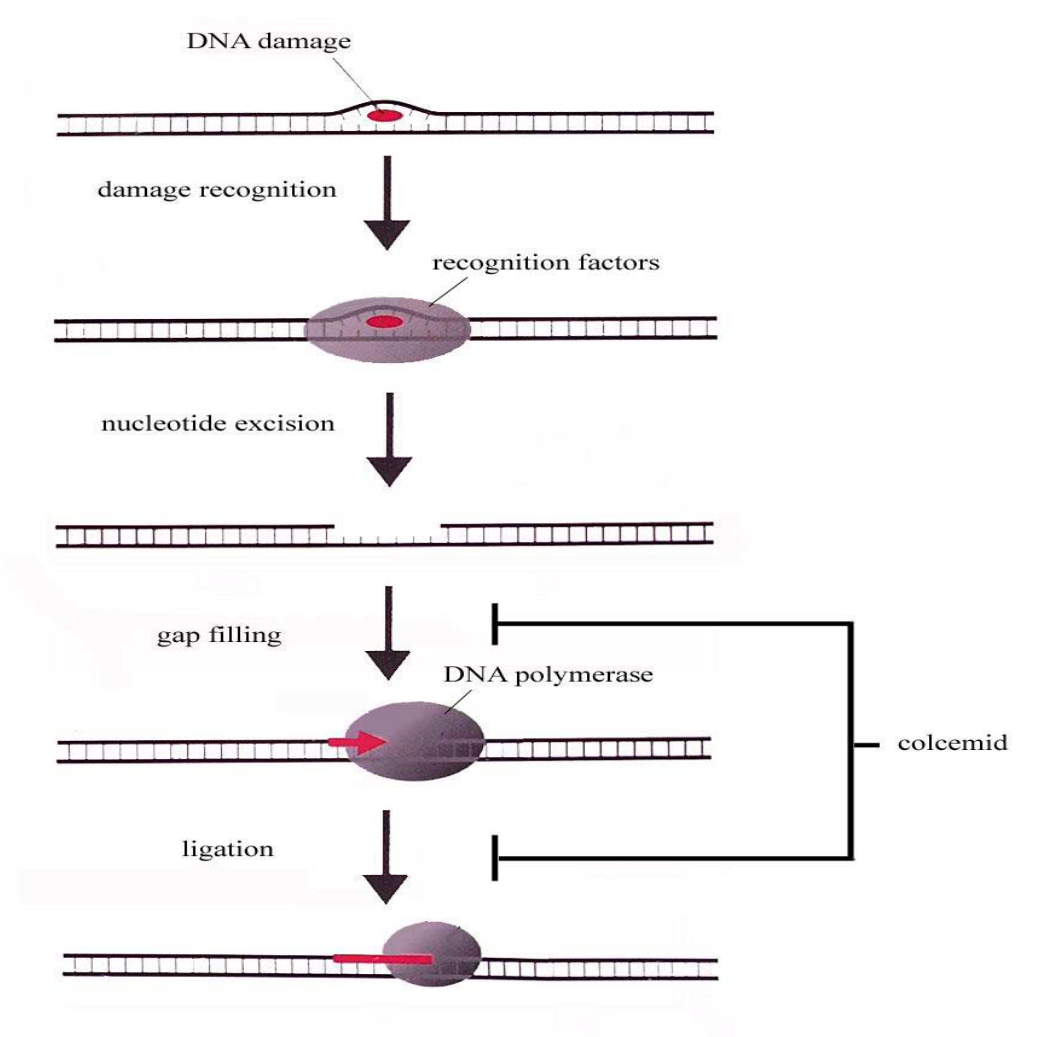

% of dead cell
40
30
20
10
0
*
*
1
2
3
4
5
6
7
8
UV
Xray
cisplatin
Colcemid
—
+
—
+
—
+
—
+

Fig 6


DNA damage
damage recognition
recognition factors
nucleotide excision
gap filling
DNA polymerase
colcemid
ligation