Acta Medica Okayama

Volume 56, *Issue* 1 2002 *Article* 3

FEBRUARY 2002

Neoadjuvant treatment with docetaxel and the effects of irradiation for human ovarian adenocarcinoma and cervical squamous cell carcinoma in vitro.

Shinako Araki[*] Yasunari Miyagi[†] Kunihiro Kawanishi[‡]
Junko Yamamoto[**] Atsushi Hongo[††] Junichi Kodama[‡‡]
Mitsuo Yoshinouchi[§] Takafumi Kudo[¶]

[*]Okayama University,
[†]Okayama Red Cross General Hospital,
[‡]Okayama University,
[**]Okayama University,
[††]Okayama University,
[‡‡]Okayama University,
[§]Okayama University,
[¶]Okayama University,

Neoadjuvant treatment with docetaxel and the effects of irradiation for human ovarian adenocarcinoma and cervical squamous cell carcinoma in vitro.*

Shinako Araki, Yasunari Miyagi, Kunihiro Kawanishi, Junko Yamamoto, Atsushi Hongo, Junichi Kodama, Mitsuo Yoshinouchi, and Takafumi Kudo

Abstract

The in vitro radiosensitizing effects of docetaxel have been reported, but the DNA damage caused by the irradiation after docetaxel exposure has not been investigated. In this study, the authors attempted to evaluate the radiosensitizing effects in terms of cell survival and DNA single-strand breaks in a human ovarian adenocarcinoma cell line (known as line BG-1) and a human cervical squamous cell carcinoma cell line (known as line SiHa). The cell lines were exposed to various concentrations of docetaxel (from 2.27 x 10(-3) to 2.27 microg/ml) to investigate the cytocidal effects by colony-formation assay. DNA single-strand breaks after exposure to 2.27 microg/ml of docetaxel for 30 min or 100 min were measured by the alkaline-elution assay. The remarkable cytotoxicity of docetaxel followed by irradiation was observed when concentrations were greater than 2.27 x 10(-2) microg/ml in both cell lines. The combination of docetaxel and irradiation appears to be supraadditive. The DNA single-strand breaks induced by the irradiation were enhanced in both cell lines (BG-1; $P < 0.01$, SiHa; $P < 0.05$). The synergistic cytocidal effect cannot be explained quantitatively only by the single-strand breaks.

KEYWORDS: docetaxel, DNA single-strand break, radiosensitizer

---

*PMID: 11873939 [PubMed - indexed for MEDLINE]

Acta Med. Okayama, 2002
Vol. 56, No. 1, pp. 13-18

Acta Medica Okayama

http://www.lib.okayama-u.ac.jp/www/acta/

Original Article

Neoadjuvant Treatment with Docetaxel and the Effects of Irradiation for Human Ovarian Adenocarcinoma and Cervical Squamous Cell Carcinoma *In Vitro*

Shinako Araki[a], Yasunari Miyagi[b]*, Kunihiro Kawanishi[a], Junko Yamamoto[a], Atsushi Hongo[a], Junichi Kodama[a], Mitsuo Yoshinouchi[a], and Takafumi Kudo[a]

[a]*Department of Obstetrics and Gynecology, Okayama University Medical School, Okayama 700-8558, Japan, and*
[b]*Department of Obstetrics and Gynecology, Okayama Red Cross General Hospital, Okayama 700-8607, Japan*

The *in vitro* radiosensitizing effects of docetaxel have been reported, but the DNA damage caused by the irradiation after docetaxel exposure has not been investigated. In this study, the authors attempted to evaluate the radiosensitizing effects in terms of cell survival and DNA single-strand breaks in a human ovarian adenocarcinoma cell line (known as line BG-1) and a human cervical squamous cell carcinoma cell line (known as line SiHa). The cell lines were exposed to various concentrations of docetaxel (from 2.27×10^{-3} to 2.27 μg/ml) to investigate the cytocidal effects by colony-formation assay. DNA single-strand breaks after exposure to 2.27 μg/ml of docetaxel for 30 min or 100 min were measured by the alkaline-elution assay. The remarkable cytotoxicity of docetaxel followed by irradiation was observed when concentrations were greater than 2.27×10^{-2} μg/ml in both cell lines. The combination of docetaxel and irradiation appears to be supraadditive. The DNA single-strand breaks induced by the irradiation were enhanced in both cell lines (BG-1; $P < 0.01$, SiHa; $P < 0.05$). The synergistic cytocidal effect cannot be explained quantitatively only by the single-strand breaks.

Key words: docetaxel, DNA single-strand break, radiosensitizer

Docetaxel as well as paclitaxel represents a novel class of anti-neoplastic drugs that bind specifically to microtubules. This binding promotes the polymerization of tubulin into stable microtubules and inhibits depolymerization. The action on microtubules and the mechanism of radiopotentiation of docetaxel appears to be not the same as that of paclitaxel either quantitatively or qualitatively [1]. Docetaxel causes an inactivation of cdc2 kinase resulting in G2M arrest [2, 3]. The taxanes arrest cells at the G2M phase of the cell cycle, and the subsequent apoptosis is dose-dependent [4, 5]. This disruption of the normal equilibrium ultimately leads to cell death [6]. In contrast, apoptosis is induced without G2 M arrest in low-dose exposure to taxanes [7, 8]. Because cells in mitosis are considerably more sensitive to radiation than cells in interphase [9], taxoids can under some circumstances increase the effects of irradiation. There have been some reports regarding the radiosensitizing effects of docetaxel [2, 10-12]. Though DNA damage is the target of irradiation, the radiation-induced DNA damage after docetaxel treatment has not investigated. In this study, we investigated the actions of docetaxel as a radiosensitizer in terms of cytotoxicity and DNA damage.

Received May 9, 2001; accepted July 23, 2001.
*Corresponding author. Phone: +81-86-222-8811; Fax: +81-86-222-8841
E-mail: ymiyagi@mac.com (Y. Miyagi)

Materials and Methods

Cell growth and maintenance. The human

ovarian adenocarcinoma cell line BG-1 and the human squamous cell carcinoma cell line derived from uterine cervical cancer, SiHa, were grown. BG-1 was cultured in modified McCoy's 5A medium (GIBCO, Grand Island, NY, USA) supplemented with 0.05% L-glutamine (GIBCO), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate (GIBCO) [13], and 10% v/v heat-inactivated fetal calf serum (GIBCO). SiHa was grown in Dulbecco's Modified Eagle medium (D-MEM)(GIBCO), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate (GIBCO), and 10% v/v heat-inactivated fetal calf serum (GIBCO) [14]. These cells were grown in 75-cm^2 polystyrene tissue-culture flasks (Becton-Dickinson, Franklin Lakes, NJ, USA) in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cultures were generated as a single stratum. The BG-1 and SiHa cells were taken off with 0.17% trypsin (GIBCO) and added to 20 mg% EDTA (Fisher). Exponentially proliferations of these cells grown for 3–4 days were used.

Irradiation. Cells at 37 °C were irradiated with x-rays of 5.0 Gy (125 kV, 15 mA) with an MBR-1520 irradiator, (Hitachi-Medico, Tokyo, Japan). The cells were then cooled immediately in ice.

Drug exposure. Docetaxel (RP 56976) powder, kindly provided by Rhône-Poulenc-Rorer Co. (France), was diluted with a culture media containing sera at 37 °C. The final concentration of the solution contained less than 0.1% ethanol, which has been found to be nontoxic to cell lines (data not shown). The solution was warmed to 37 °C, and all exposures were performed at a consistent temperature. The docetaxel concentrations ranged from 2.27×10^{-3} to 2.27 μg/ml according to clinical pharmacokinetic parameters [15]. The exposure times were 30 and 100 min. After exposure, the cells were rinsed 3 times with docetaxel-free subculture medium at 37 °C. The rinsed cells were immediately irradiated. In the cytotoxicity experiment regarding 30-min docetaxel exposure, however, the rinsed cells were irradiated either immediately or after 70-min incubation at 37 °C.

DNA analysis. The cells were treated in 2.27 μg/ml of docetaxel and then rinsed. DNA crosslinking was measured with the alkaline-elution assay [16–19] described by Miyagi *et al.* with modifications [13]. The cells (3×10^6) for each channel were loaded on polycarbonate filters 47 mm in diameter with a pore size of 2 μm (Costar Scientific Co., Cambridge, MA, USA) in Swinnex filter holders (Millipore Co., Bedford, MA, USA). The cells were then lysed in 2 M NaCl (Wako, Osaka, Japan), 0.2% N-lauroylsarcosine (Sigma Chemical Co., St. Louis, MO, USA), and 0.04 M EDTA (Ishizu Seiyaku Ltd., Osaka, Japan; pH 10.0) to remove most of the cell proteins, membranes, and RNA. The filter was then rinsed with 0.76% Na_4-EDTA (Ishizu Seiyaku Ltd.) and 33 ml of a solution of 0.02 M EDTA (Sigma Chemical Co.) and 20% tetrapropylammonium hydroxide, pH 12.3, (Tokyokasei, Tokyo, Japan). The cells were then overlaid in the dark and pumped through a peristaltic pump (Gilson Medical Electronics S.A., Villiers-le-Bel, France) to a fraction collector (Advantec Toyo, Osaka, Japan) at a rate of 0.0389 ml/min. Ten fractions were collected at 90-min intervals for 15 h. At least 3 channels were used for each cell-suspension specimen. Each fraction was mixed with 0.08 M KH_2PO_4 (Katayamakagaku, Osaka, Japan) and Hoechst 33258 dye (Sigma Chemical Co.). DNA detection was then performed with a fluorometric assay [20–22]. Each DNA analysis was repeated 3 times. We did not use proteinase K in the lysis solution because disposal by proteinase K appeared to cause no differences in the elution profiles in our preliminary experiments (data not shown).

The amount of DNA from cells on the filter was calculated as a percentage of the amount retained after elution. The single-strand scission factor in the seventh fraction (SSSF7) was calculated by the following formula [20]:

$$SSSF7 = -\ln[Ri/Ru],$$

in which Ri is the percentage of irradiated DNA retained on the filter in fraction 7, and Ru is the percentage of unirradiated DNA retained on the filter in fraction 7.

Colony formation. A clony-formation assay was used as a survival assay in this study. Briefly, the cells suspended in media with 10% v/v fetal calf serum were seeded into culture dishes 60 mm in diameter (Becton-Dickinson). The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 10 days. The colonies were then stained with 0.1% crystal violet. Colonies of > 32 cells were counted. The percentage of cell growth for each treatment was calculated by dividing the number of colonies in the treated culture by the number of colonies in the untreated culture. Each colony-formation assay was repeated between 3 and 5 times.

Statistical analysis. The values were analyzed by the two-way analysis of variance test, the

Friedman test, and the unpaired t-test.

Results

The cytotoxicity of docetaxel alone for the cell lines showed dose-dependency (Fig. 1). There were no differences in the cytotoxicity in terms of exposure time in the BG-1 cells. In contrast, the SiHa cells showed more toxicity after 100-min expoure than after 30-min expoure.

The cytotoxicity of 5 Gy of irradiation in the BG-1 and SiHa cell lines was $1.42 \pm 0.04\%$ and $1.39 \pm 0.06\%$ (mean $\pm$ S.D.), respectively. The remarkable cytotoxicity of docetaxel followed by irradiation was observed when concentrations were greater than 2.27×10^{-2} μg/ml in both cell lines (Fig. 2). There were significant differences with regard to concentration ($P < 0.005$) in both cell

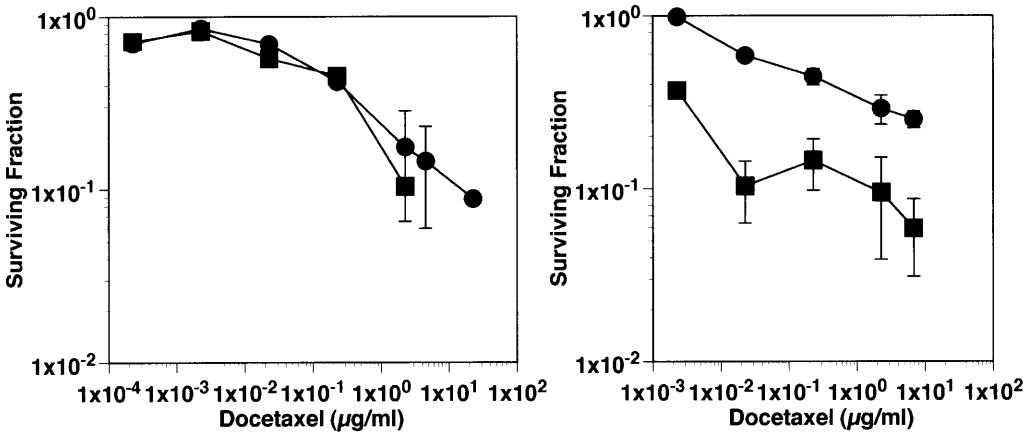

Fig. 1 Colony-formation efficiency of docetaxel exposure with no following irradiation in the BG-1 (left panel) and SiHa cell lines (right panel). The SiHa cells showed more toxicity after exposure for 100-min than for 30-min. The values represent the mean $\pm$ SEM. Error bars (SE) are shown where they are larger than the symbols. (●, 30-min exposure; ■, 100-min exposure)

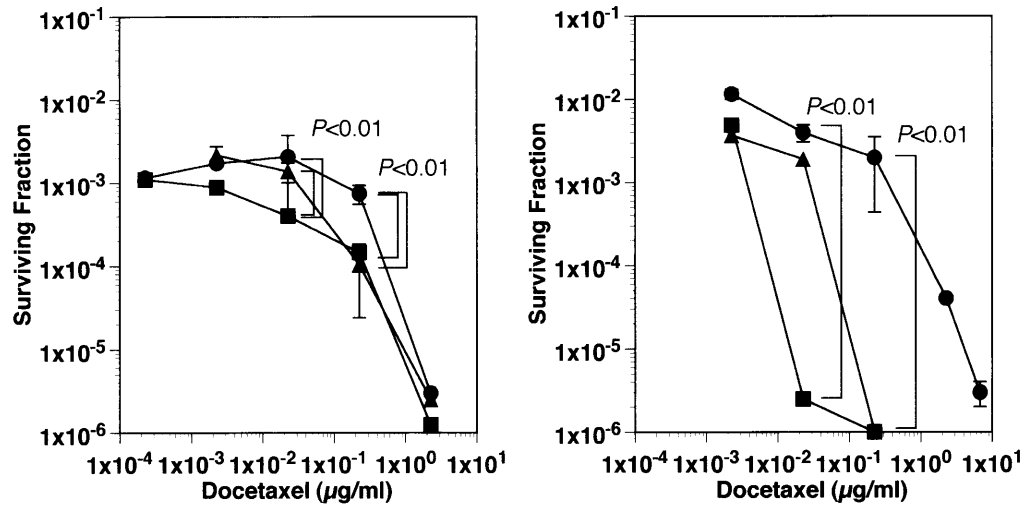

Fig. 2 Colony-formation efficiency of three types of docetaxel exposure in the BG-1 (left panel) and SiHa cell lines (right panel). Significant differences in terms of concentration in both cell lines ($P < 0.005$, respectively). In both cell lines, the 70-min incubation enhanced the cytocidal effects at 0.27 μg/ml of docetaxel ($P < 0.01$, respectively), and the 100-min exposure showed more cytocidal effects with over 0.27 μg/ml of docetaxel ($P < 0.01$, respectively). The values represent the mean $\pm$ SEM. Error bars (SE) are shown where they are larger than the symbols. (●, 30-min exposure; ▲, 30-min exposure followed by drug-free incubation for 70 min; ■, 100-min exposure)

lines.

The expected cytotoxicity value of docetaxel followed by irradiation, if this combination effect is additive, is considered to be a result of the survival fraction of docetaxel alone multipled by the survival fraction of 5 Gy for irradiation alone. The effect ratio, which is the experimental value of the combination procedure divided by the expected cytotoxicity value, would be more than one if the combination effect is supraadditive. Fig. 3 shows that the combination of docetaxel and irradiation is supraadditive.

Fig. 4 demonstrates that the DNA single-strand

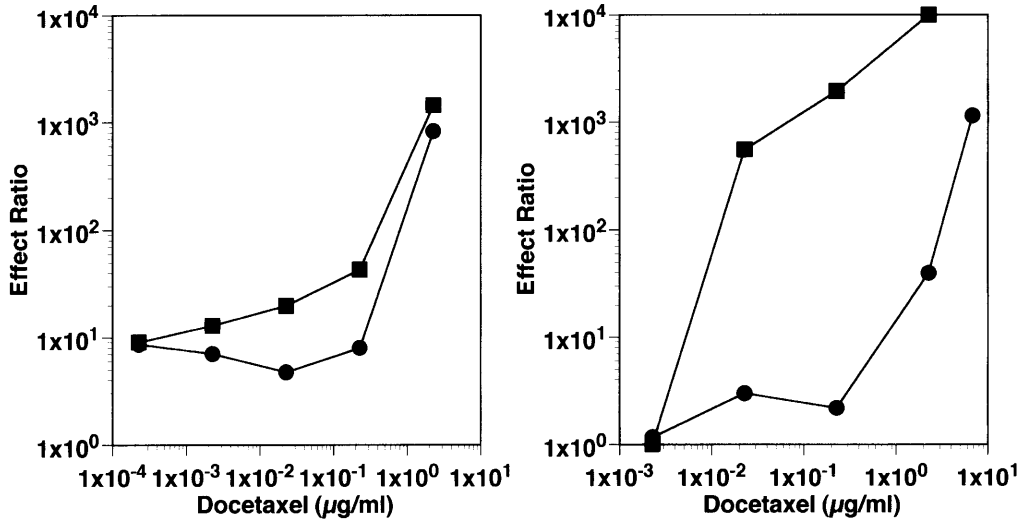

Fig. 3 The effect ratios, which represent the experimental values for docetaxel exposure followed by irradiation divided by the expected cytotoxicity value, which is the result of the survival fraction of docetaxel alone multipled by the survival fraction of 5 Gy of irradiation alone, for the BG-I (left panel) and SiHa cell lines (right panel) are shown. The effect ratio is one if the combination procedure is additive. The combination of docetaxel exposure followed by the irradiation results in a higher ratio, making the combination supuraadditive. (●, 30-min exposure; ■, 100-min exposure)

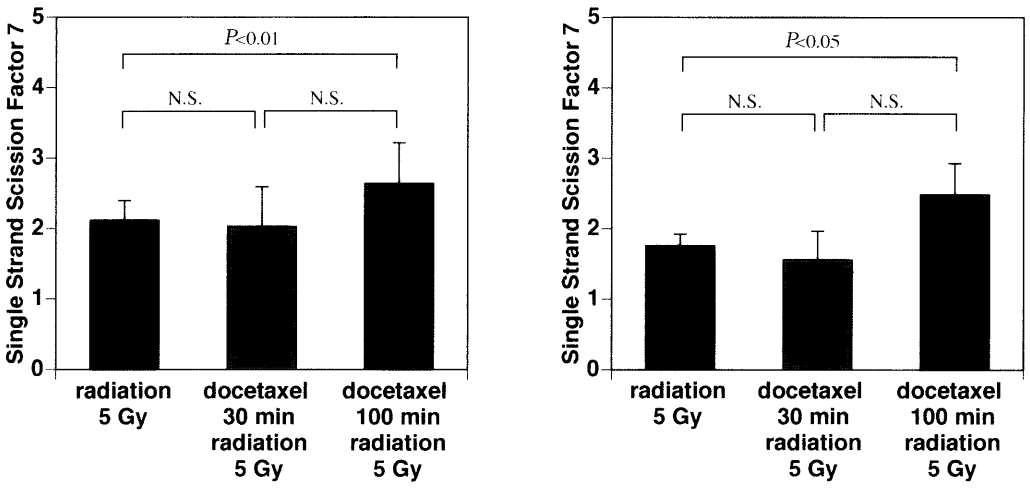

Fig. 4 The single-strand scission factor in the seventh fraction for the 3 types of treatment on the BG-I (left panel) and SiHa cell lines (right panel). The values represent the mean ± SEM. Prior to irradiation, cells were exposed to 2.27 μg/ml of docetaxel. It appears that the DNA single-strand breaks induced by the irradiation were enhanced by exposure to docetaxel for 100 min in both cell lines (BG-I; $P < 0.01$, SiHa; $P < 0.05$). In contrast, 30 min of docetaxel exposure resulted in no enhancement effect on DNA single-strand breaks.

breaks induced by irradiation are enhanced by 2.27 μg/ml of docetaxel exposure for 100 min in both cell lines (BG-1; $P < 0.01$, SiHa; $P < 0.05$). Exposure to the same concentrations of docetaxel for 30 min, however, resulted in no enhancement effect. The value of the single-strand scission factor in the seventh fraction showed no differences between the 2 cell lines.

Discussion

Docetaxel was found to enhance the cytotoxicity caused by irradiation in both the BG-1 and SiHa cell lines in this study. This finding for not only paclitaxel [23-27] but also docetaxel [2-12, 28] has been reported in other cell lines. The combination procedure showed a supraadditive effect. The higher the docetaxel concentrations the cells were exposed to, the greater the effective the radiation-enhanced cytotoxicity became, as shown in Fig. 3.

An isobologram is generally used for evaluating the combination of the 2 kinds of treatments. The survival fractions of the lowest concentrations of docetaxel and 5 Gy of irradiation were approximately $< 1\%$ and 1.4%, respectively, however. In addition, the radiation dose was fixed at 5 Gy. Therefore, an isobologram cannot be used to quantitatively evaluate the synergistic effect in the present study. The approximate exponential relationship between the docetaxel concentrations and the effect ratios were observed, as shown in Fig. 3.

A remarkable cytocidal effect was observed especially at higher concentrations. It appears that the exposure time of docetaxel is likely to enhance the cytocidal effects.

As such, we then investigated the mechanism of supraadditive synergistic cytocidal effects of docetaxel and irradiation in terms of DNA damage induced by the irradiation. There have been no reports regarding the relationship between radiosensitization and the radiation target DNA obstacle in terms of docetaxel, to the best of our knowledge. And because the ionizing radiation causes DNA strand breaks, we therefore measured DNA single-strand breaks by the alkaline-elution method. In this study, because the rinsed cells after docetaxel exposure were cooled at ice-cold temperatures immediately after the irradiation treatment, the observed number of single-strand breaks, which were formed in direct response to the irradiation with no possibility of subsequent repair of DNA damage, were considered to be a direct indicator of the ionizing radiation. The docetaxel exposure for 100 min increased the number of DNA single-strand breaks, as shown in Fig. 4. The value of the single-strand scission factor in the seventh fraction in the 100-min exposure followed by 5 Gy of radiation was equivalent to 6.5 Gy of irradiation, which was calculated by a calibration curve in a preliminary experiment (data not shown). Moreover, 2.27 μg/ml of docetaxel exposure with no following irradiation did not cause DNA single-strand breaks in a preliminary experiment (data not shown). Therefore, the radiosensitizing effects of docetaxel for 100 min were equivalent to 1.5-Gy enhancement. In contrast, exposure to docetaxel for 30 min did not produce radiosensitizing effects. Though there is a significant synergistic cytocidal effect produced by the combination, the number of the DNA single-strand breaks cannot explain the phenomena quantitatively. The DNA double strand breaks induced by radiation are said to cause cell death. Therefore, it may be possible that some unknown mechanisms may be inducing more double-strand than single-strand breaks when cells are treated with docetaxel prior to irradiation. It is therefore of importance to analyze DNA double-strand breaks as well as other factors such as the cell cycle, metabolic changes, and apoptosis induction, in order to investigate the mechanism.

Docetaxel can act to some extent as a radiosensitizer. It would therefore be useful to wait some time prior to radiation if docetaxel is clinically used as a radiosensitizer. Docetaxel induces increases in the number of DNA single-strand breaks, but the synergistic cytocidal effects cannot be explained by single-strand breaks alone.

References

1. Gueritte-Voegelein F, Guenard D, Lavelle F, Le Goff MT, Mangatal L and Potier P: Relationships between structure of taxol analogues and their antimitotic activity. J Med Chem (1991) **34**, 992-998.
2. Pradier O, Rave-Fränk M, Lehmann J, Lüke E, Boghun O, Hess CF and Schmidberger H: Effects of docetaxel in combination with radiation on human head and neck cancer cells (ZMK-1) and cervical squamous cell carcinoma cells (CaSki). Int J Cancer (2001) **91**, 840-845.
3. Nehme A, Varadarajan P, Sellakumar G, Gerhold M, Niedner H, Zhang Q, Lin X and Christen RD: Modulation of docetaxel-induced apoptosis and cell cycle arrest by all-trans retinoic acid in prostate cancer cells. Br J Cancer (2001) **84**, 1571-1576.
4. Choy H: Taxanes in combined modality therapy for solid tumors. Crit Rev Oncol Hematol (2001) **37**, 237-247.
5. Goncalves A, Braguer D, Carles G, Andre N, Prevot C and Briand C: Caspase-8 activation independent of CD95/CD95-L interaction during paclitaxel-induced apoptosis in human colon cancer cells (HT29-D4). Biochem Pharmacol (2000) **60**, 1579-1584.

6. Ringel I and Horwitz SB: Studies with RP 56976 (taxotere): A semisynthetic analogue of taxol. J Natl Cancer Inst (1991) **83**, 288–291.
7. Lin HL, Liu TY, Chau GY, Lui WY and Chi CW: Comparison of 2-methoxyestradiol-induced, docetaxel-induced, and paclitaxel-induced apoptosis in hepatoma cells and its correlation with reactive oxygen species. Cancer (2000) **89**, 983–994.
8. Amorino GP, Hamilton VM and Choy H: Enhancement of radiation effects by combined docetaxel and carboplatin treatment *in vitro*. Radiat Oncol Investig (1999) **7**, 343–352.
9. Terashima R and Tolmach LJ: X-ray sensitivity and DNA synthesis in synchronous populations of HeLa cells. Science (1963) **140**, 490–492.
10. Distelmans W and Storme G: The effect of pretreatment with docetaxel (taxotere; RP56976) on irradiated subcutaneous MA 16/C murine tumors. J Exp Clin Cancer Res (1999) **18**, 167–172.
11. Milas L, Milas MM and Mason KA: Combination of taxanes with radiation: Preclinical studies. Semin Radiat Oncol (1999) **9** (2 Suppl 1), 12–26.
12. Hennequin C, Giocanti N and Favaudon V: Interaction of ionizing radiation with paclitaxel (Taxol) and docetaxel (Taxotere) in HeLa and SQ20B cells. Cancer Res (1996) **56**, 1842–1850.
13. Miyagi Y, Zhang H and Wheeler KT: Radiation-induced DNA damage in tumors and normal tissues: IV. Influence of proliferation status and cell type on the formation of oxygen-dependent DNA damage in cultured cells. Radiat Res (1997) **148**, 29–34.
14. Friz F, Ikuo K, Toyoro O and Yohei I: Studies on a New Human Cell Line (SiHa) Derived from Carcinoma of Uterus. I. Its Establishment and Morphology (35091) Carcin Cell Line (1970) **135**, 543–545.
15. Extra JM, Rousseau F, Bruno R, Clavel M, Le Bail N and Marty M: Phase I and pharmacokinetic study of Taxotere (RP 56976, NSC 628503) given as a short intravenous infusion. Cancer Res (1993) **53**, 1037–1042.
16. Kohn KW, Erickson LC, Ewig RA and Friedman CA: Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry (1976) **15**, 4629–4637.
17. Ewig RA and Kohn KW: DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis (2-chloroethyl)-1-nitrosourea, and other nitrosoureas. Cancer Res (1977) **37**, 2114–2122.
18. Fornace AJ Jr and Kohn KW: DNA-protein cross-linking by ultraviolet radiation in normal human and xeroderma pigmentosum fibroblasts. Biochim Biophys Acta (1976) **435**, 95–103.
19. Zwelling LA, Kohn KW, Ross WE, Ewig RA and Anderson T: Kinetics of formation and disappearance of a DNA cross-linking effect in mouse leukemia L1210 cells treated with cis-and trans-diamminedichloroplatinum (II). Cancer Res (1978) **38**, 1762–1768.
20. Zhang H and Wheeler KT: Radiation-induced DNA damage in tumors and normal tissues. I. Feasibility of estimating the hypoxic fraction. Radiat Res (1993) **136**, 77–88.
21. Swarts SG, Nelson GB, Wallen CA and Wheeler KT: Radiation-induced cytotoxicity, DNA damage and DNA repair: Implications for cell survival theory. Radiat Environ Biophys (1990) **29**, 93–102.
22. Wheeler KT, Hickman R, Nelson GB, Moore SK and Wallen CA: Relationship between DNA damage, DNA repair, metabolic state and cell lethality. Radiat Environ Biophys (1992) **31**, 101–115.
23. Tishler RB, Schiff PB, Geard CR and Hall EJ: Taxol: A novel radiation sensitizer. Int J Radiat Oncol Biol Phys (1992) **22**, 613–617.
24. Steren A, Sevin BU, Perras J, Angioli R, Nguyen H, Guerra L and Averette HE: Radiosensitization by taxol of a human ovarian cancer cell line. Proc Am Soc Clin Oncol (1992) **33**, 552.
25. Choy H, Rodriguez FF, Koester S, Hilsenbeck S and Von Hoff D: Investigation of taxol as a potential radiation sensitizer. Cancer (1993) **71**, 3774–3778.
26. Liebmann J, Cook JA, Fisher J, Teague D and Mitcheell JB: In vitro studies of Taxol as a radiation sensitizer in human tumor cells. J Natl Cancer Inst (1994) **86**, 441–446.
27. Creane M, Seymour CB, Colucci S and Mothersill C: Radiobiological effects of docetaxel (Taxotere): A potential radiation sensitizer. Int J Radiat Biol (1999) **75**, 731–737.
28. Talwar N and Redpath JL: Schedule dependence of the interaction of radiation and Taxol in HeLa cells. Radiat Res (1997) **148**, 48–53.