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ORIGINAL ARTICLE

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The vitamin D_3 analog, ILX-23-7553, enhances the response to Adriamycin and irradiation in MCF-7 breast tumor cells

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Abstract Ionizing radiation and the anthracycline antibiotic, Adriamycin, generally fail to promote a primary apoptotic response in experimental breast tumor cell lines. Similarly, the primary response of breast tumor cells to vitamin D_3 ($1,25(OH)_2D_3$) and vitamin D_3 analogs such as EB 1089 is growth inhibition. Previous studies have demonstrated that pretreatment of MCF-7 breast tumor cells with vitamin D_3 or EB 1089 can increase sensitivity to both Adriamycin and irradiation. *Purpose*: The capacity of the vitamin D_3 analog, ILX 23-7553, to enhance the antiproliferative and cytotoxic effects of Adriamycin or irradiation and to promote apoptosis in MCF-7 breast tumor cells was assessed in the present study. *Results*: Pretreatment of MCF-7 cells with ILX 23-7553 followed by Adriamycin or irradiation decreased viable cell numbers by 97% and 93%, respectively. Cell numbers were reduced by 56%, 74% and 75% by ILX 23-7553, Adriamycin and irradiation alone. Pretreatment with ILX 23-7553 also shifted the dose response curve for clonogenic survival, increasing sensitivity to Adriamycin 2.5-fold and sensitivity to radiation fourfold. In addition, ILX 23-7553 pretreatment conferred sensitivity to Adriamycin- or irradiation-induced DNA fragmentation and resulted in morphological changes indicative of apoptotic cell death in MCF-7 cells. Statistical analysis demonstrated that ILX 23-7553 interacts additively and not synergistically with both Adriamycin and irradiation. *Conclusions*: ILX 23-7553 enhances the effects of Adriamycin and irradiation in MCF-7 breast tumor cells by decreasing viable cell numbers, reducing clonogenic survival and inducing apoptotic cell death. Current studies are focused on elucidating the mechanisms underlying the induction of apoptosis as well as understanding the nature of the interactions between ILX 23-7553 and Adriamycin or irradiation.

Key words ILX 23-7553 · Adriamycin · Radiation · Breast tumor cells · Apoptosis

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Introduction

Breast cancer remains the most common malignant disease among middle-aged women in the United States. Surgery and radiotherapy are thought to be effective in the treatment of localized tumors while adjuvant therapy involving drugs such as Adriamycin is utilized with the goal of eliminating metastatic cells within the breast as well as at other sites which are relatively inaccessible [11]. Despite these intensive efforts, metastatic breast cancer remains an essentially incurable disease with a high incidence of recurrence. Although there are multiple reasons for disease recurrence, one contributory factor may be the existence of populations of breast tumor cells that are resistant to chemotherapy and radiotherapy due to their failure to undergo apoptotic cell death. Tumor cells of hematopoietic or lymphatic origin as well as colorectal and melanoma cells respond to radiation and other DNA-damaging agents with characteristic programmed cell death (apoptosis) [5, 26, 27, 44]. Human breast tumor cells, however, tend to be refractory to apoptotic cell death in response to treatments which induce DNA damage such as the topoisomerase II inhibitor, Adriamycin, and ionizing radiation [13, 14, 18, 19, 38, 39, 40].

Ravid et al. [28] have reported that the response of MCF-7 breast tumor cells to relatively high concentrations of Adriamycin can be enhanced by $1,25(OH)_2D_3$ and Wang et al. [37] have demonstrated that

$1,25(OH)_2D_3$ sensitizes breast tumor cells to a variety of chemotherapeutic drugs by increasing cell death. Recent reports from our laboratory also indicate that the relatively nontoxic vitamin D_3 analogue, EB 1089 [9, 12], enhances the response of breast tumor cells to Adriamycin and ionizing radiation, also promoting the induction of apoptosis [34, 35]. In the current studies, we determined the effects on cell survival and apoptosis of combining either ionizing radiation or Adriamycin with the $1,25(OH)_2D_3$ analog ILX 23-7553.

Materials and methods

Materials

The p53 wild-type MCF-7 human breast tumor cell line was obtained from NCI (Frederick, Md.). ILX 23-7553 was generously provided by ILEX Products (San Antonio, Tx.). MCF-7 cells transfected with a dominant negative temperature-sensitive mutant p53 (p53-143 val-ala) were generously provided by Drs. S. Fan and E.M. Rosen of the Long Island Jewish Medical Center/Albert Einstein College of Medicine [1]. The p53 mutant T-47D breast tumor cell line was obtained from ATCC. RPMI-1640 and supplements were obtained from GIBCO Life Technologies (Gaithersburg, Md.). Reagents used for the terminal transferase end labeling (TUNEL) assay (terminal transferase, reaction buffer, and fluorescein-dUTP) were purchased from Boehringer Mannheim (Indianapolis, Ind.). All other reagents used in the study were analytical grade.

Cell culture

All cell lines were grown from frozen stocks in basal RPMI-1640 medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine and penicillin/streptomycin at 37ºC under a humidified atmosphere containing 5% CO_2. All experiments were conducted using approximately 10^4 cells/cm^2 on day 0 with the use of time-equivalent and concentration-equivalent controls. The results shown are averages of two to three experiments.

Trypan blue exclusion

The response of cells treated with Adriamycin (1 μ*M*) for 2 h and irradiation (10 Gy) was assessed 72 h after treatment by trypan blue exclusion. The response of cells treated with ILX 23-7553 (200 n*M*) for 72 h was evaluated 72 h after removal of ILX 23-7553. To evaluate the combined effects of Adriamycin or irradiation with ILX 23-7553 (200 n*M*), cells were treated with ILX 23-7553 for 72 h prior to Adriamycin (1 μ*M*) or irradiation (10 Gy) and allowed to grow for an additional 72 h. Cells were harvested using trypsin, stained with 0.4% trypan blue and trypan blue-negative cells were counted under a phase contrast microscope.

Clonogenic analysis

MCF-7 cells were treated with ILX 23-7553 (200 n*M*), Adriamycin or radiation alone and with ILX-23-7553 preceding various doses of Adriamycin or radiation. For analysis of additivity, MCF-7 cells were treated with various doses of ILX 23-7553 (0–200 n*M*) in combination with a single dose of either Adriamycin (20 n*M*) or irradiation (2.5 Gy). Cells were trypsinized immediately following Adriamycin or radiation treatment under sterile conditions and plated in triplicate in six-well cell culture plates at approximately 1000 cells for each condition. After 7–10 days, the cells were fixed with 100% methanol, air-dried for 1–2 h and stained with 0.1% crystal violet. Groups of 50 or more cells were counted as colonies.

TUNEL

The method of Gavrieli et al. [15] was utilized as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and non-adherent cells. The cells were fixed and the fragmented DNA in cells undergoing apoptosis was detected using an In Situ Cell Death Detection Kit (Boehringer-Mannheim). In this assay, the fragmented DNA in individual cells was end-labeled using fluorescein-dUTP at strand breaks by the enzyme terminal transferase. The slides were then washed, mounted in Vectashield and photographed using a Nikon fluorescent microscope. For quantitation, fluorescent cells were scored positive for DNA fragmentation. These results are expressed as the number of fluorescent cells divided by the total number of cells in each field.

Cell morphology

Cells were washed 72 h after Adriamycin or irradiation exposure, and cytocentrifuged onto microscope slides. The cells were stained with Wright-Giemsa stain and photographed under a Nikon light microscope.

Synergism vs additivity

The predicted responses for the colony forming assay were determined using the following additivity model: $y = \exp(B0 + B1x1 + B2x2)$ where y is the predicted response, x_1 is the dose of either irradiation (10 Gy) or Adriamycin (n*M*), x_2 is the dose of ILX 23-7553 (n*M*), B_0 is an unknown parameter associated with the intercept, B_1 is an unknown parameter associated with the slope of x_1, and B_2 is an unknown parameter associated with the slope of x_2. Parameter estimates were found using a generalized least squares criterion for nonlinear models. A constant variance was assumed across the dose range of all four compounds. The Gauss-Newton iterative algorithm was used in PROC NLIN in SAS (version 6.12) to find parameter estimates. To compare the observed response at each combination point to that predicted under the hypothesis of additivity, a prediction interval was used following the method of Gennings et al. [16]. An overall test for additivity [17] was based on testing the hypothesis that the mean response under the hypothesis of additivity is the true mean response. The estimated responses under the hypothesis of additivity were provided by the additivity model. The estimated responses for the true means were provided by the sample means of each mixture group.

Results

Influence of ILX 23-7553 pretreatment followed by Adriamycin or ionizing radiation on viable cell numbers and clonogenic survival

Previous studies in this laboratory have demonstrated that the vitamin D_3 analog, EB 1089, enhances the response of MCF-7 breast tumor cells to both Adriamycin and irradiation [34, 35]. In order to determine if pretreatment with the vitamin D_3 analog ILX 23-7553 (200 n*M*) could similarly influence the response of breast tumor cells to Adriamycin or irradiation, viable cell numbers were determined by trypan blue exclusion 72 h

after MCF-7 cells were treated with Adriamycin (1 μ*M*) or irradiation (10 Gy) alone or with ILX 23-7553 pretreatment. Figure 1 indicates that viable cell numbers were decreased by 60%, 74% and 75%, respectively, in cells treated with ILX 23-7553, Adriamycin and irradiation alone compared to growth of untreated controls. ILX 23-7553 pretreatment significantly increased the effectiveness of both Adriamycin and irradiation. Cells treated with ILX 23-7553 in combination with Adriamycin or irradiation showed reductions of 97% and 93%, respectively, in viable cell numbers.

In separate studies, it was determined that, even with a 24-h delay between removal of the ILX-23-7553 and exposure of the cells to Adriamycin or irradiation, there was a substantial increase in the degree of growth inhibition following treatment with the combinations (Table 1). Table 1 further indicates that when treatment with ILX-23-7553 was combined with Adriamycin or irradiation in p53-mutated T-47D cells or in MCF-7 cells with a dominant negative mutant p53 [1], the degree of growth inhibition was significantly less than that observed in the p53 wild-type MCF-7 cells. In fact, the growth-inhibitory effects of ILX23-7553 combined with radiation did not differ significantly from that of radiation alone in T-47D cells.

The next series of studies were performed to determine if the enhanced effectiveness of ILX-23-7553 in combination with Adriamycin or irradiation on MCF-7 cell growth translated into effects on clonogenic survival. MCF-7 cells were exposed to Adriamycin concentrations ranging from 1 to 100 n*M* or 0.5 to 5 Gy ionizing radiation with and without 200 n*M* ILX 23-7553 pretreatment. Figure 2A demonstrates that the concentration of Adriamycin required to reduce clonogenic survival by 50% was decreased by approximately 2.5-fold by ILX 23-7553 pretreatment, declining from about 38 n*M* to about 14 n*M*. The sensitivity of MCF-7 cells to ionizing radiation was more profoundly influenced by ILX 23-7553 than was sensitivity to Adriamycin such that there was a fourfold reduction in the dose of irradiation required to produce a 50% decrease in clonogenic survival from 1 Gy to 0.25 Gy (Fig. 2B). Thus, ILX 23-7553 in combination with either Adriamycin or irradiation decreased clonogenic survival and viable cell numbers in MCF-7 cells more effectively than treatment with any of the agents alone.

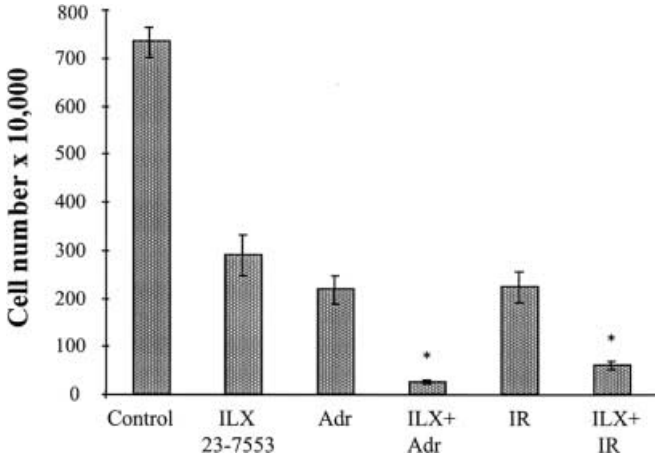

Fig. 1 Influence of ILX 23-7553 pretreatment followed by Adriamycin or ionizing radiation on viable numbers of MCF-7 cells. Cells were treated with 200 n*M* ILX 23-7553 for 72 h followed by exposure to 1 μ*M* Adriamycin (acute exposure) or 10 Gy ionizing radiation. Viable cell numbers were determined by trypan blue exclusion 72 h after exposure to Adriamycin or irradiation. Cell numbers were decreased by 60%, 74% and 75%, respectively, after treatment with ILX 23-7553, Adriamycin or irradiation alone. ILX 23-7553 pretreatment followed by Adriamycin or ionizing radiation decreased viable cell numbers by 97% and 93%, respectively. Asterisk indicates values significantly different from ADR, IR or ILX alone

Influence of ILX 23-7553 pretreatment followed by Adriamycin or irradiation on the incidence of apoptotic cell death

Previous work has shown that neither Adriamycin nor irradiation induces a primary apoptotic cell death response in MCF-7 cells [13, 14, 18, 19, 38, 39, 42]. In order to determine if the enhanced ability of Adriamycin and irradiation to decrease cell viability and clonogenic survival when preceded by ILX 23-7553 treatment could be attributed to an increased incidence of apoptosis, the TUNEL assay was performed. Figure 3 indicates that DNA fragmentation indicative of apoptotic cell death was not induced by Adriamycin, irradiation or ILX 23-7553 alone. In contrast, ILX 23-7553 pretreatment followed by Adriamycin or irradiation increased the number of cells with DNA fragmentation to 15% and 7% cells, respectively (Fig. 3B).

In addition to DNA fragmentation, apoptosis is characterized by morphological changes including condensation of the cell cytoplasm and the formation of apoptotic bodies. An analysis of cell morphology was performed to assess whether ILX 23-7553 pretreatment followed by Adriamycin or irradiation could induce

Table 1 Comparison of the effects of ILX 23-7553 in combination with either Adriamycin or ionizing radiation on viable cell numbers in MCF-7 cells where exposure to Adriamycin or radiation occurred immediately after removal of ILX 23-7553, in MCF-7 cells exposed to Adriamycin or irradiation 24 h after removal of ILX 23-7553, in p53 mutant T-47D cells and in MCF-7 cells expressing a dominant negative temperature-sensitive p53 gene. Values are viable cell numbers expressed as a percent of controls 72 h after exposure to Adriamycin or radiation

Treatment	MCF-7	MCF-7, 24-h delay	T-47D	p53 mutant MCF-7
ILX 23-7553	38.9 ± 1.62	58.0 ± 3.66	56.6 ± 6.25	52.0 ± 1.65
Adriamycin	25.0 ± 2.27	42.9 ± 5.27	27.5 ± 3.45	38.9 ± 11.03
Irradiation	27.3 ± 2.16	39.5 ± 4.31	37.1 ± 9.33	50.0
ILX + Adriamycin	3.2 ± 1.0	12.8 ± 8.37	15.5 ± 1.27	20.6 ± 0.25
ILX + irradiation	8.4 ± 0.48	18.0 ± 6.72	34.2 ± 8.51	20.1 ± 5.72

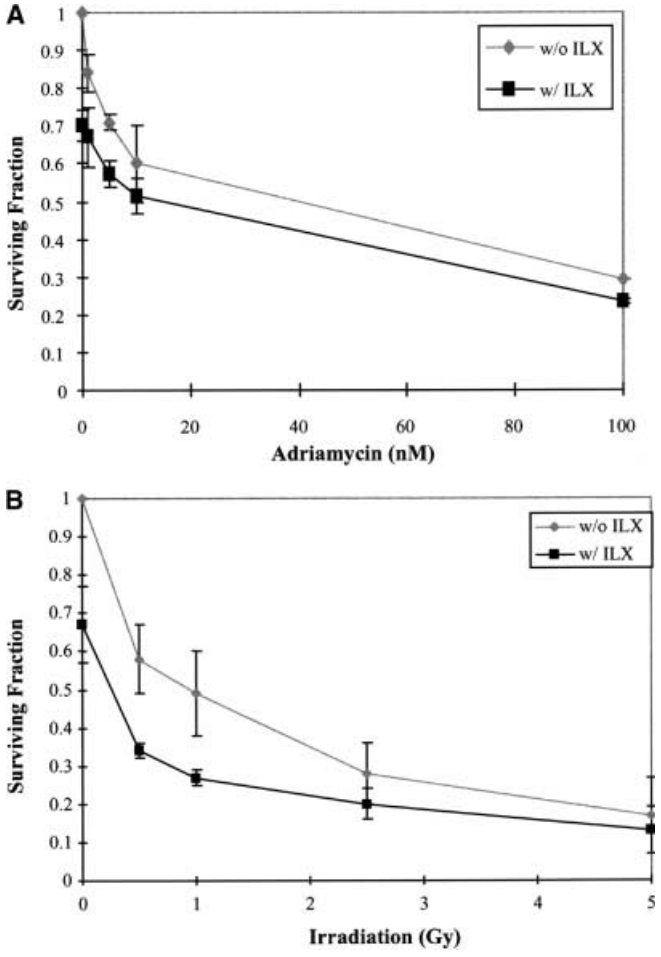

Fig. 2A, B Clonogenic survival of MCF-7 cells pretreated with ILX 23-7553 followed by various doses of Adriamycin (**A**) or ionizing radiation (**B**). Cells were treated with 200 n*M* ILX 23-7553 for 72 h followed by exposure to 1–100 n*M* Adriamycin or 0.5–5.0 Gy radiation. Colony numbers were determined 7–10 days after Adriamycin or irradiation treatment. ILX 23-7553 pretreatment produced 2.5-fold and fourfold decreases, respectively, in the doses of Adriamycin and irradiation required to reduce clonogenic survival by 50%

such morphological changes. As shown in Fig. 4, apoptotic cells which appeared dark and shrunken compared to normal cells were observed after exposure to ILX 23-7553 in combination with both Adriamycin and irradiation. Treatment with Adriamycin, irradiation or ILX 23-7553 alone, however, did not produce cells with this apoptotic morphology.

ILX 23-7553 interacts additively with both Adriamycin and irradiation

ILX 23-7553 in combination with Adriamycin or irradiation produced DNA fragmentation, apoptotic morphology, decreased cell viability and decreased clonogenic capacity of MCF-7 cells. However, the shift in the dose response curves shown in Fig. 2 may simply reflect additive interactions between ILX 23-7553 and Adriamycin or irradiation. We therefore determined whether the observed effects were occurring through a synergistic or additive interaction between ILX 23-7553 and Adriamycin or irradiation. The results of two colony forming assays, one in which a single dose of ILX 23-7553 (200 n*M*) was combined with multiple doses of Adriamycin (1–100 n*M*) and another in which a single dose of Adriamycin (20 n*M*) was combined with various doses ILX 23-7553 (0–200 n*M*), were analyzed statistically and compared with those predicted by the statistical model of additivity (Table 2). The observed surviving fractions were determined not to be significantly different from those predicted by the model of additivity ($P = 0.461$). Therefore, the assumption of additivity was not rejected and ILX 23-7553 and Adriamycin were concluded to interact additively and not synergistically.

Statistical analysis was also performed to evaluate the ILX 23-7553 and irradiation combination. Results predicted by the model of additivity were compared with those observed using a single dose of ILX 23-7553 (200 n*M*) and various doses of irradiation (0.5–5 Gy) as well as a single dose of irradiation (2.5 Gy) and multiple doses of ILX 23-7553 (Table 3). Similar to the Adriamycin combination, the observed surviving fractions were not significantly different from those predicted using the model of additivity ($P = 0.196$). Therefore, the assumption of additivity was not rejected and ILX 23-7553 and irradiation were concluded to interact additively.

Discussion

Previous studies have confirmed that clinically relevant doses of Adriamycin and ionizing radiation fail to induce apoptotic cell death in human breast tumor cells [13, 14, 18, 19, 38, 39, 42] and it is possible that this lack of apoptotic response may be partially responsible for tumor recurrence [18]. In this context, Liu et al. [24] have recently demonstrated the importance of apoptosis in the response of breast tumor cells to chemotherapy in a syngeneic mouse model.

There is evidence that differentiation therapy combined with conventional chemotherapy and/or radiotherapy is effective in causing apoptosis in tumors of hematopoietic origin [3]. Additionally, the effectiveness of radiation in human solid tumor cells can be enhanced with differentiating agents such as *N*-methylformamide and hexamethylene bisacetamide [2, 4]. Vitamin D_3 and its analogs which are relatively nontoxic compounds have been shown to differentiate leukemic, lymphoma and breast tumor cells [3, 12, 33]. Studies by Light et al. [23] as well as Cho et al. [8] have demonstrated a potentiating effect on growth inhibition in MCF-7 cells and murine squamous carcinoma cells by combining $1,25(OH)_2D_3$ with platinum drugs. Furthermore, recent work by other investigators has demonstrated that

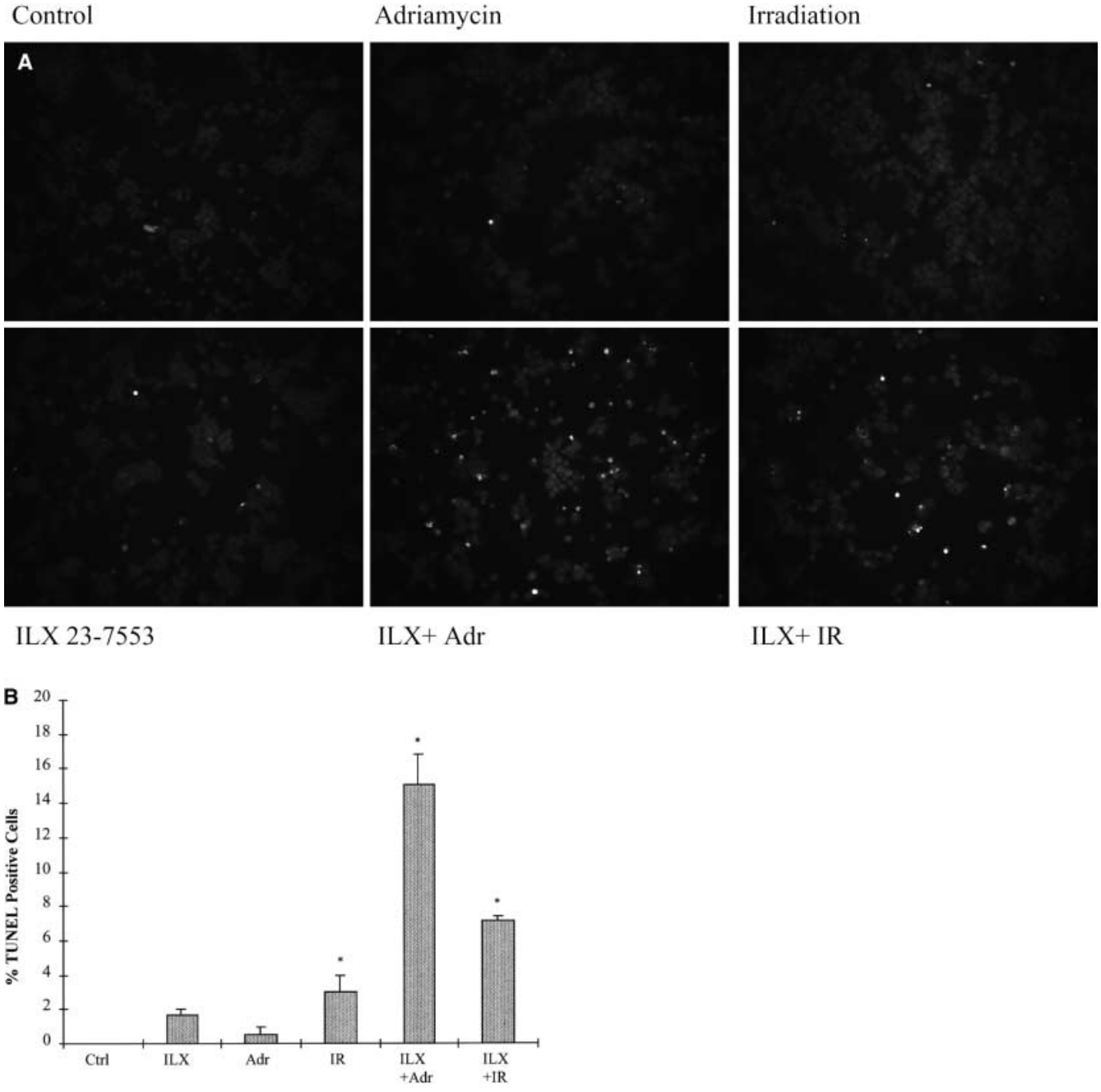

Fig. 3 **A** Enhancement of DNA fragmentation by ILX 23-7553 in combination with Adriamycin and ionizing radiation. Cells were cytospun onto glass slides after pretreatment with 200 n*M* ILX 23-7553 followed by exposure to 1 μ*M* Adriamycin or 10 Gy radiation. **B** Percent TUNEL-positive cells. DNA fragmentation indicative of apoptosis was evident in 14% and 7% of cells, respectively, pretreated with ILX 23-7553 followed by Adriamycin or irradiation. Percent TUNEL-positive cells is the fraction of fluorescent cells. Asterisk indicates values significantly different from control

$1,25(OH)_2D_3$ enhances the response to Adriamycin in breast tumor cells [28, 37] while it has been shown in this laboratory that the vitamin D_3 analog EB 1089 enhances the response of p53 wild-type breast tumor cells to radiation [34] and Adriamycin [35]. Consequently, in attempting to circumvent the functional resistance to apoptosis observed in breast tumor cells, we investigated the effectiveness of exposing MCF-7 breast tumor cells to ILX 23-7553 in combination with either Adriamycin or irradiation.

MCF-7 cells were pretreated with ILX 23-7553 prior to Adriamycin or irradiation exposure. As reported by Ravid et al. [28], at least 24 h of pretreatment is necessary for the effectiveness of the combinations to be expressed. We believe that this interval may be necessary to allow time for vitamin D_3 and its analogs to alter the ratio between insulin-like growth factor and insulin-like growth factor binding proteins [22] and/or to influence the levels of the epidermal growth factor receptor [43]. The combination of ILX-23-7553 with either Adriamy-

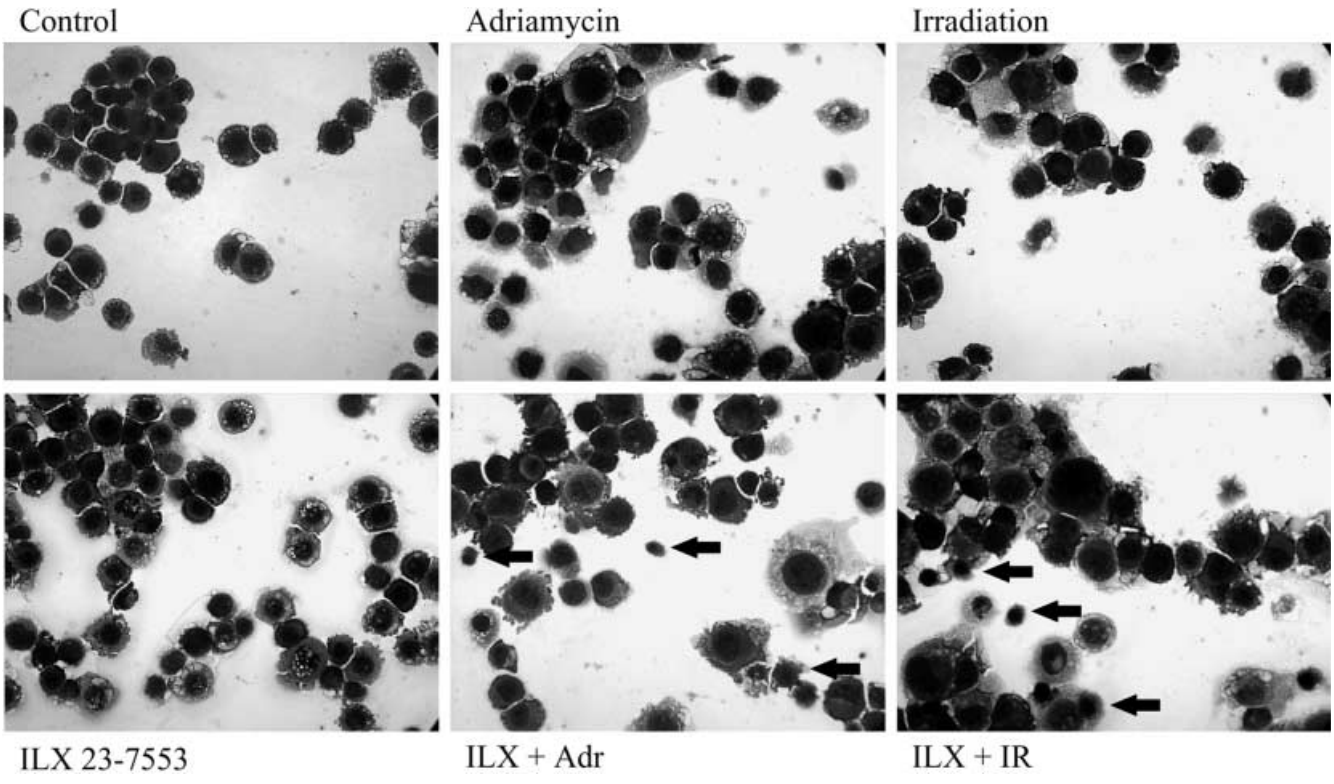

Fig. 4 Influence of ILX 23-7553 in combination with Adriamycin or ionizing radiation on cell morphology. Cells were cytospun onto glass slides after pretreatment with 200 n*M* ILX 23-7553 followed by exposure to 1 μ*M* Adriamycin or 10 Gy irradiation. Cells pretreated with ILX 23-7553 followed by Adriamycin or irradiation show morphological changes indicative of apoptosis. *Arrows* indicate apoptotic bodies

cin or radiation resulted in reductions in viable cell numbers and in clonogenic survival that significantly exceeded those produced by any of the individual treatments. These studies recapitulate our previous findings using EB 1089. Interestingly, while EB 1089 and ILX-23-7553 appeared to be similarly effective in reducing viable cell numbers in combination with Adriamycin or irradiation, somewhat more pronounced effects on clonogenic survival were evident with EB 1089.

As in our previous studies involving EB 1089 [34, 35], the effects of ILX23-7553 in combination with either Adriamycin or radiation were less pronounced in p53 mutant cells. The fact that the combinations appear to be somewhat more effective in the temperature-sensitive p53 mutant MCF-7 cells than in T-47D cells may relate to lack of complete suppression of wild-type p53 function which could have occurred as a result of temperature fluctuations during the course of these experiments.

In addition to the effects on cell numbers and clonogenic survival, ILX 23-7553 in combination with Adriamycin or irradiation appeared to enhance DNA fragmentation and produce morphological changes indicative of apoptotic cell death. The mechanistic basis for apoptosis induction is currently under investigation. In preliminary experiments, we failed to detect a blockade to the Adriamycin-induced increase in MAP kinase activity [29, 35], to alter the increase in levels of the cyclin-dependent kinase inhibitory protein $p21^{waf1/cip1}$ induced by Adriamycin [6], to influence levels of the proapoptotic Bax protein [30] or levels of IKB alpha, a protein that is degraded to permit activation of the antiapoptotic activity of NF kappa B [32, 45].

The role of apoptosis in the enhancement of breast tumor cell sensitivity to Adriamycin and irradiation by vitamin D_3 and vitamin D_3 analogs remains uncertain. Our findings suggest that the potentiation of the effectiveness of Adriamycin by ILX 23-7553 in breast tumor cells may occur, at least in part, through the promotion of apoptotic cell death in otherwise apoptosis-resistant cells. This concept is supported by the fact that the potentiating effects of ILX23-7553 are more pronounced in the p53 wild-type cells. However, the fact that the effect of ILX 23-7553 in combination with either Adriamycin or irradiation are merely additive raises the issue of whether the promotion of apoptosis is critical in this cell line as reduced clonogenic survival and viable cell numbers could occur through other mechanisms including necrosis and reproductive cell death [7, 21, 36]. Furthermore, the combination of ILX-23-7553 with Adriamycin has a more pronounced effect on apoptosis than ILX23-7553 in combination with radiation, yet sensitization of the cells is greater in the case of radiation than in the case of Adriamycin. This observation suggests a limited role for apoptosis in potentiating loss of

Table 2 Additive interaction between ILX 23-7553 and Adriamycin. Results of the colony forming assay were compared with values predicted using a statistical model of additivity. Observed and predicted values were not significantly different (mean $P = 0.46$); therefore, the assumption of additivity was not rejected

Drug combination		Observed mean response (surviving fraction)	Predicted response under additivity (surviving fraction)	95% Prediction intervals under additivity
Adriamycin (n*M*)	ILX 23-7553 (n*M*)			
20	50	0.58	0.63	[0.27, 0.99]
20	100	0.50	0.56	[0.19, 0.93]
1	200	0.67	0.53	[0.14, 0.93]
5	200	0.58	0.51	[0.12, 0.91]
10	200	0.52	0.49	[0.10, 0.88]
20	200	0.46	0.44	[0.05, 0.83]
100	200	0.24	0.20	[0, 0.60]

Table 3 Additive interaction between ILX23-7553 and radiation. Results of the colony forming assay were compared with values predicted using a statistical model of additivity. Observed and predicted values were not significantly different (mean $P = 0.196$); therefore, the assumption of additivity was not rejected

Treatment combination		Observed mean response (surviving fraction)	Predicted response under additivity (surviving fraction)	95% Prediction intervals under additivity
Radiation (Gy)	ILX 23-7553 (n*M*)			
2.5	50	0.23	0.29	[0, 0.38]
0.5	200	0.34	0.51	[0, 0.61]
1.0	200	0.27	0.41	[0.14, 0.88]
2.5	200	0.20	0.22	[0.06, 0.77]
5.0	200	0.13	0.08	[0, 0.55]

cell survival in this experimental system – which would be consistent with reports that promotion of apoptosis accelerates cell death without affecting clonogenic survival [20, 25]. On the other hand, recent work in our laboratory (unpublished) has demonstrated that cells that undergo prolonged growth arrest after Adriamycin or ionizing radiation exposure have the capacity to recover reproductive capacity, while work in a number of laboratories has shown the phenomenon of repopulation after irradiation [10, 31, 41]. Consequently, the promotion of apoptosis could interfere with disease recurrence by preventing the survival of cells which might otherwise repopulate the breast (or other sites to which the tumors may have metastasized).

In summary, the initial impact of utilizing nontoxic vitamin D_3 analogs such as ILX-23-7553 could be to reduce the dose of conventional chemotherapeutic protocols as well as radiotherapy required for a given level of cell killing. In addition, the long-term effectiveness of these treatment protocols could be enhanced by the promotion of apoptotic cell death thereby preventing tumor cell recovery from the toxic injury and limiting disease recurrence. Therefore, nontoxic analogs of $1,25(OH)_2D_3$ could prove to be beneficial in clinical protocols by enhancing both the immediate and the long-term effectiveness of chemotherapeutic drugs and radiation against breast tumor cells.

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References

1. Andres JL, Fan S, Turker GJ, Wang Ji-A, Twu N-F, Yuan R-Q, Lamszus K, Goldberg ID, Rosen EM (1998) Regulation of BRCA1 and BRCA2 expression in human breast cancer cells by DNA damaging agents. Oncogene 16:2229
2. Arundel C, Bock S, Brock WA, Tofilon PJ (1987) Radiosensitization of primary human tumor cell cultures by N-methylformamide. J Radiat Oncol Biol Phys 13:753
3. Bhatia U, Traganos F, Darzynkiewic Z (1995) Induction of cell differentiation potentiates apoptosis triggered by prior exposure to DNA damaging drugs. Cell Growth Differ 6:937
4. Bill CA, Vines CM, Garrett KC, Yamada K, Tofilon PJ (1990) Enhancement of radiosensitivity of two human tumour cell lines by hexamethylene bisacetamide. Br J Cancer 61:563
5. Bracey TS, Miller JC, Preece A, Paraskeva C (1995) Gamma-radiation-induced apoptosis in human colorectal adenoma and carcinoma cell lines can occur in the absence of wild type p53. Oncogene 10:2391
6. Canman CE, Kastan MB (1998) Small contribution of G1 checkpoint control manipulation to modulation of p53-mediated apoptosis. Oncogene 16:957
7. Chang WP, Little JB (1991) Delayed reproductive cell death in X-irradiated Chinese hamster ovary cells. Int J Radiat Biol 60:483
8. Cho YL, Christensen C, Saunders DE, Lawrence WD, Deppe G, Malviya VK, Malone JM (1991) Combined effects of $1,25(OH)_2D_3$ and platinum drugs on the growth of MCF-7 cells. Cancer Res 51:2848
9. Colston KW, Mackay AG, James SY, Binderup L, Chandler S, Coombes RC (1992) EB1089, a new vitamin D analog that inhibits the growth of breast cancer cells in vivo and in vitro. Biochem Pharmacol 44: 2273
10. Denekemp J (1973) Changes in the rate of repopulation during multi-fraction irradiation of mouse skin. Br J Radiol 46:381
11. DeVita VT, Hellman S, Rosenberg SA (1998) Cancer: principles and practice of oncology. Lippincott, Philadelphia

12. Elsten E, Linker-Israeli M, Said J, Umiel T, deVos S, Shintaku P, Heber D, Binderup L, Uskokovic M, Koeffler HP (1995) 20-epi-Vitamin D3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. Cancer Res 55:2822
13. Fornari FA, Jarvis WD, Grant S, Orr MS, Randolph JK, White FKH, Mumaw VR, Lovings ET, Freeman RH, Gewirtz DA (1994) Induction of differentiation and growth arrest associated with nascent (nonligosomal) DNA fragmentation and reduced c-myc expression in MCF-7 human breast tumor cells after continuous exposure to a sublethal concentration of doxorubicin. Cell Growth Differ 5:723
14. Fornari FA, Jarvis WD, Orr MS, Randolph JK, Grant S, Gewirtz DA (1996) Growth arrest and non-apoptotic cell death associated with the suppression of c-myc expression in MCF-7 breast tumor cells following acute exposure to doxorubicin. Biochem Pharmacol 51:931
15. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death In situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493
16. Gennings C, Schwartz P, Carter WH Jr, Simmons JE (1997) Detection of departures from additivity in mixtures of many chemicals with a threshold model. J Agric Biol Environ Stat 2:198
17. Gennings C, Carter WH Jr, Campain J, Bal D, Yarn R (2000) Statistical analysis of interactive cytotoxicity in human epidermal keratinocytes following exposure to a mixture of four metals. J Agric Biol Environ Stat (in press)
18. Gewirtz DA (2000) Growth arrest and cell death in the breast tumor cell in response to ionizing radiation and chemotherapeutic agents which induce DNA damage. Breast Cancer Res Treat 62:223
19. Gewirtz DA, Randolph JK, Chawla J, Orr MS, Fornari FA (1998) Induction of DNA damage, inhibition of DNA synthesis and suppression of c-myc expression by the anthracycline analog, idarubicin (4-demethoxy-daunorubicin) in the MCF-7 breast tumor cell line. Cancer Chemother Pharmacol 41:361
20. Han JW, Dionne CA, Kedersha NL, Goldmacher VS (1997) p53 status affects the rate of onset but not the overall extent of doxorubicin-induced cell death in rat-1 fibroblasts constitutively expressing c-myc. Cancer Res 57:176
21. Hendry JH, West CML (1997) Apoptosis and mitotic cell death: their relative contributions to normal tissue and radiation response. Int J Radiat Biol 71:709
22. Koga M, Eisman JA, Sutherland RL (1998) Regulation of epidermal growth factor receptor levels by 1,25 dihydroxy vitamin D3 in human breast cancer cells. Cancer Res 15:2734
23. Light BW, Yu WD, McElwain MC, Russell, DM, Trump DL, Johnson CS (1997) Potentiation of cisplatin antitumor activity using a vitamin D analogue in a murine squamous cell carcinoma model system. Cancer Res 57:3759
24. Liu R, Page C, Beidler DR, Wicha MS, Nunez G (1999) Overexpression of Bcl-x (L) promotes chemotherapy resistance of mammary tumors in a syngeneic mouse model. Am J Pathol 155:1861
25. Lock RB, Stribinskiene L (1996) Dual modes of death induced by etoposide in human epithelial cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. Cancer Res 56:4006
26. Radford IJ, Murphy TK, Radlev JM, Ellis SL (1994) Radiation response of mouse lymphoma and melanoma cells. Part II. Apoptotic death is shown in all cell lines examined. Int J Radiat Biol 65:217
27. Ramqvist T, Magnusson KP, Wang Y, Szekely L, Klein G, Wiman KG (1993) Wild-type p53 induces apoptosis in a Burkit Lymphoma (BL) line that carries mutant p53. Oncogene 8:1495
28. Ravid A, Rocker D, Machlenikin A, Rotem C, Hochman A, Kessler-Icekson G, Liberman UA, Koren R (1999) 1,25-Dihydryoxyvitamin D3 enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage. Cancer Res 59:862
29. Reardon DB, Contessa JN, Mikkelsen RB, Valerie K, Amir C, Dent P, Schmidt-Ullrich RK (1999) Dominant negative EGFR-CD533 and inhibition of MAPK modify JNK1 activation and enhance radiation toxicity of human mammary carcinoma cells. Oncogene 18:4755
30. Reed JC, Miyashita T, Takayama S, Wang HG, Sato T, Krajewski S, Aime-Sempe C, Bodrug S, Kitada S, Hanada M (1996) BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 60:23
31. Schmidt-Ullrich RK, Mikkelsen RB, Dent P, Todd DG, Valerie K, Kavanaugh BD, Contessa JN, Rorrer WK, Chen PB (1997) Radiation-induced proliferation of human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. Oncogene 15:1911
32. Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM, Sonenshein GE (1997) Aberrant nuclear factor kappa B/Rel expression and pathogenesis of breast cancer. J Clin Invest 100:2952
33. Studinski GP, McLane JA, Uskokovic MR (1993) Signalling pathways for vitamin D induced differentiation: implications for therapy of proliferative and neoplastic diseases. Crit Rev Eukaryot Gene Expr 3:279
34. Sundaram S, Gewirtz DA (1999) The vitamin D3 analog EB 1089 enhances the response of human breast tumor cells to radiation. Radiat Res 152:479
35. Sundaram S, Chaudhry M, Reardon D, Gewirtz DA (2000) EB 1089 enhances the antiproliferative and apoptotic effects of Adriamycin in MCF-7 breast tumor cells. Breast Cancer Res Treat 63:1
36. Szumiel I (1994) Review: ionizing radiation induced cell death. Int J Radiat Biol 66:329
37. Wang Q, Yang W, Uytingco MS, Christakos S, Wieder R (2000) 1,25-Dihydroxyvitamin D3 and all-trans retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. Cancer Res 60:2040
38. Watson NC, Di Y-M, Orr MS, Fornari FA, Randolph JK, Magnet KJ, Jain PT, Gewirtz DA (1997) The influence of ionizing radiation on proliferation, c-myc expression and the induction of apoptotic cell death in two breast tumor cell lines differing in p53 status. Int J Radiat Biol 72:547
39. Whitacre CM, Berger NA (1997) Factors affecting topotecan induced programmed cell death: adhesion protects cells from apoptosis and impairs cleavage of polyADP-ribose polymerase. Cancer Res 57:2157
40. Wilson JW, Wakeling AE, Morris ID, Hickman JA, Dive C (1995) MCF-7 human mammary adenocarcinoma cell death in vitro in response to hormone withdrawal and DNA damage. Int J Cancer 51:502
41. Withers HR, Taylor JMG, Maciejewski B (1988) The hazard of accelerated tumor clonogenic repopulation during radiotherapy. Acta Oncol 27:131
42. Wosikowski K, Regis JT, Roby RW, Alvarez M, Buters JT, Gudas JM, Bates SE (1995) Normal p53 status and function despite the development of drug resistance in human breast cancer cells. Cell Growth Differ 6:139
43. Xie SP, Pirianov G, Colston KW (1999) Vitamin D analogues suppress IGF-1 signaling and promote apoptosis in breast cancer cells. Eur J Cancer 35:1717
44. Yonish-Rouach E, Reznitsk D, Lotem, J, Sachs L, Kimchi A, Oren M (1991) Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-2. Nature 352:345
45. Yu XP, Bellido T, Manolagas SC (1995) Downregulation of NF-kappa B protein levels in activated human lymphocytes by 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci USA 92:10990